INVESTIGATION OF THE COELIAC IMMUNOGENICITY OF CEREAL PEPTIDES AND THEIR QUANTIFICATION USING MONOCLONAL ANTIBODIES

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INVESTIGATION OF THE COELIAC IMMUNOGENICITY OF CEREAL PEPTIDES AND THEIR QUANTIFICATION USING MONOCLONAL ANTIBODIES

A thesis presented for the degree of Doctor of Philosophy

by

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ABSTRACT

Coeliac disease (CD) is a chronic, immune-mediated, small intestinal gluten-sensitive enteropathy affecting approximately 1% of individuals in the Europe and the U.S. The condition is exacerbated by the consumption of food containing wheat gluten, rye secalin and barley hordein. The keystone of CD treatment is strict compliance to a gluten-free diet. Designed foods for coeliac sufferers are mainly based on purified wheat starch that may contain traces of gluten. Hence, a sensitive and specific assay system is required to ensure the purity and safety of commercially available gluten-free foods. Such a system is based on the use of monoclonal antibodies (MAbs) raised against coeliac toxic gluten peptides. Several peptides have been identified in rye and barley that are thought to be potentially immunogenic and in turn CD toxic. An ω-gliadin/ C-hordein peptide (QPFPQPEQPFPW) and a rye secalin-derived peptide (QPFPQPQQPIPQ) have been identified from the literature as potentially CD toxic. They were assessed for CD in vitro immunogenicity and used to raise MAbs.

We wished to investigate the in vitro responses of small intestinal gluten sensitive T cell lines to the described peptides. We have sought to develop MAbs to these coeliac toxic peptides as a prelude to developing improved assays to quantify the gluten content of foods for individuals with CD. In parallel to this work, using the available MAbs previously developed by our group, we proposed to design a specific Cocktail ELISA assay for gluten analysis. This would potentially provide an assay with improved specificity and sensitivity for CD toxic gluten proteins and peptides.

The CD immunogenicity of ω-gliadin/ C-hordein peptide and a rye secalin-derived peptide were assessed with T cell proliferation assays. These were undertaken by incorporation of ^{3}H-thymidine to measure the immune response of the peptides and prolamin fractions to CD gluten sensitive small intestinal T cell lines. Balb c mice that received a gluten-free diet were immunised with PPD (tuberculin protein) conjugated to the coeliac toxic peptides described above. The gluten content in purified wheat starch which is used to make commercially available gluten-free foods was quantified using a unique cocktail ELISA by combining two of established MAbs raised against known CD toxic peptides.

We found that the ω-gliadin/ C-hordein peptide and a rye secalin-derived peptide triggered positive responses in most of the CD small intestinal T cell lines specific to peptic-tryptic (PT) digested wheat gluten (n=9), barley hordein (n=7) and rye secalin (n=2), confirming their coeliac immunogenicity. The murine

immunisations and fusions were successful. However, IgM antibodies were generated by the hybridomas to the gluten peptides that could not be used for gluten quantification. We found that we can quantify both CD toxic gliadin and glutenin in separate assays, simultaneously. Our method for gluten quantification could represent an improvement in the specificity of gluten quantification, since it measures CD toxic wheat proteins, including both gliadin and glutenin, rather than the currently accepted method of multiplying the gliadin content by two to yield a value for the total gluten content. However, we found that combining the MAbs in a single Cocktail assay yielded lower values compared to the summation of the individual quantified components.

The ω-gliadin/ C-hordein peptide and a rye-derived peptide are immunogenic and were recognised by CD gluten sensitive T cell lines. Balb c mice were successfully immunised with these immunogenic peptides. The resultant hybridomas secreted IgM rather than IgG antibodies to the peptides. The former of which could not be used to develop immunological assays for gluten quantification. Characterisation of cocktail ELISAs with more than one MAb combination to gluten protein demonstrated lower values of gluten than the summation of simultaneous quantification of the individual components. We concluded that CD toxic gliadin and glutenin need to be measured individually and the value of both proteins be added together to quantify the gluten content of foods designed for individual with CD.

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LISTS OF ABBREVIATIONS

AGA	Anti gliadin antibodies
APC	Antigen presenting cells
ASM	Autologous serum medium
BSA	Bovine serum albumin
CD	Coeliac disease
$CaCl_2$	Calcium chloride
CFA	Complete Freund's adjuvant
CPM	Counts per minute
Da	Dalton
DH	Dermatitis herpetiformis
DMSO	Dimethyl sulphoxide
ECH	Enterocyte cell height
ELISA	Enzyme-linked immunosorbent assay
EMA	Antiendomysial antibodies
FCS	Fetal calf serum
GFD	Gluten free diet
^3H	Tritiated thymidine
HAT	Hypoxanthine, aminopterin, thymidine
HLA	Human leukocyte antigen
HMW	High molecular weight
hTTG	Antihuman tissue transglutaminase antibody
HRP	Horseradish peroxidase
IBS	Irritable bowel syndrome
IEL	Intraepithelial lymphocytes
IFA	Incomplete Freund's adjuvant

IFN-γ	Interferon-γ
IL-2	Interleukin-2
Ig	Immunoglobulin
KH_2PO_4	Monobasic potassium phosphate
LP	Lamina propria
LMW	Low molecular weight
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
Na_2HPO_4	Sodium dibasic phosphate
OD	Optical density
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline with Tween 0.05%
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PPD	Tuberculin purified protein derivative
ppm	Parts per million
PT	Peptic-trypic digested
PTG	Peptic-tryptic gluten
PTH	Peptic-tryptic hordeins (barley)
PTS	Peptic-tryptic secalins (rye)
RPMI	Rosewell Park Memorial Institute
SEM	Standard error of the mean
SIL	Small intestinal lymphocytes
TTD	Tetanus toxoid
tTG	Tissue transglutaminase

AMINO ACIDS TABLE

Amino Acid	Single-Letter Code	Three-Letter Code	Molecular Weight (Da)
Alanine	A	ALA	89.09
Arginine	R	ARG	174.20
Asparagine	N	ASP	132.12
Aspartate	D	ASP	133.10
Cysteine	C	CYS	121.16
Glutamatic acid	E	GLU	147.13
Glutamine	Q	GLN	146.15
Glycine	G	GLY	75.07
Histidine	H	HIS	155.16
Isoleucine	I	ILE	131.18
Leucine	L	LEU	131.18
Lysine	K	LYS	146.19
Methionine	M	MET	149.21
Phenylalanine	F	PHE	165.19
Proline	P	PRO	115.13
Serine	S	SER	105.09
Threonine	T	THR	119.12
Tryptophan	W	TRP	204.23
Tyrosine	Y	TYR	181.19
Valine	V	VAL	117.15

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CHAPTER ONE: GENERAL INTRODUCTION

Coeliac disease (CD) is a chronic, auto-immune small intestinal disorder, affecting approximately 1% of the Western population. It is also known as Coeliac Sprue or gluten-sensitive enteropathy. The aberrant immune response to gluten protein developes in genetically predisposed individuals. The condition is exacerbated by the ingestion of toxic prolamins of wheat (gliadin and glutenin), barley (hordein) and rye (secalin). A minority of coeliac sufferers (5%) are susceptible to oat avenin (Lundin 2003).

The disease is initially diagnosed by the presence of abnormal IgA and IgG serology to tissue transglutaminase (tTG) and endomysial antibody. CD duodenal biopsy characterises itself by the appearance of small bowel mucosa such as villous atrophy, crypt hyperplasia and increased number of intraepithelial lymphocytes (IELs). Classical gastrointestinal symptoms include diarrhoea, abdominal distension and vomiting. CD is also associated with other health implications such as osteoporosis, dermatitis herpetiformis (DH) and increased risk of small bowel lymphoma and carcinoma. The keystone of treatment is strict lifelong compliance to a gluten free diet (GFD). The clinical and gastrointestinal manifestations improve if wheat related cereals, including rye and barley are omitted from the diet, and relapse occurs if there is consumption of gluten-containing food.

An increasing number of GF wheat alternatives are commercially available to complement the needs of quality life for CD patients. Designed foods for individuals with CD are largely based on purified wheat starch which may be contaminated by traces of wheat gluten. This includes including both gliadin and

glutenin wheat proteins. Rye and barley are CD toxic. These are known to share similar structures including the amino acid sequences resulting in the presence of CD gluten toxic epitopes. A sensitive and versatile assay system is required to ensure the purity and safety of specialised foods for individuals with CD. It is therefore important to be able quantify the amount of CD toxic fractions in such foods including those based on other Triticaea.

In this thesis, many aspects in relation to CD will be presented. Chapter One is the General Introduction. The second chapter summarises the aims of the study. The investigation of barley and rye peptides immunogenicity is discussed in Chapter Three. Chapter Four describes the development of monoclonal antibodies (MAbs) to barley and rye peptides. One aspect of this study pertains to the aim of designing an improved Cocktail Enzyme-linked immunosorbent assay (ELISA) for gluten analysis in purified wheat starch; this is described in Chapter Five. Finally, the general discussion and conclusion are presented in Chapter Six.

1.1 History of CD

The earliest description of CD was written in Greek in the 2nd Century by Aretaeus of Cappodocia**.** The term coeliac derived from the Greek word 'koiliakos', which Aretaeus recorded as the malabsorptive syndrome of chronic diarrhoea with abdominal distension (Adam 1856). He recorded that the occurrence of CD in adults was associated with incomplete digestion of food. The disease was further described and acknowledged by a paediatrician, Samuel Gee in 1887. This was the first significant description of the disease in the modern history of CD. Gee had included children in his observation, especially those between one and five years old. He stated that coeliac affection was 'a kind of

chronic indigestion which is met with in persons of all ages'. He also proposed that the condition could be treated with the benefit of diet. Gee recognised that coeliac children had problem with milk intolerance and recommended the avoidance of highly starched foods. He also described particular success with a child who was fed upon a quart of the best Dutch mussels daily, and the condition relapsed when the season was over (Gee 1888). The daily consumption of mussels treated the condition most probably due to the child's health improvement, by not ingesting gluten in his diet.

In 1924, an American physician named Dr Hass introduced a low carbohydrate banana diet for CD patients following a case of anorexia nervosa cured by the same regimen (Hass 1924). A Dutch physician, Dr. Willem Karel Dicke pioneered the wheat-free diet during World War II when the bread supply was scarce in Holland. He noticed the tremendous health improvement of his patients with reduced bread consumption. He realised that the affected children benefited noticeably when they were on wheat-free diet. He suggested that the ingestion of wheat proteins specifically, but not carbohydrates in general, were the cause of CD (Dicke 1953). The diagnosis was routinely made with faecal fat estimation by Dicke's laboratory colleagues who confirmed that the exclusion of wheat, barley, rye and oats improved the patients' clinical conditions (Dicke 1953).

1.2 The prevalence of CD

The disease prevalence was rather uncommon in the 50s indicated by the epidemiological studies by Davidson and Fountain (1950), with only 0.0125% of the populations affected in England and 0.025% in Scotland. The estimation of CD prevalence has been greatly improved with the better understanding of the disease and development of more reliable serological screening methods. The

majority of CD occurrence was noted in the European populations; the prevalence in the UK was estimated to be around 1% of the adults aged 45 to 76 (West 2003, Bingley 2004). Larger studies that involved four different European countries has shown an average of 1% prevalence; where Finland is the highest (2%), followed by Italy (1.2%), Northern Ireland (0.9%) and Germany (0.3%), respectively (Mustalahti 2010). Similar magnitude has been documented in other Caucasian populations in USA with prevalence of 1:133 (Fasano 2003). In many South American countries that are inhabited by individuals of European origin, CD has been quite common. The prevalence of CD was in Brazil was reported to be 1:214 (Oliveira 2007) whereas in Argentina, CD was found to be 1 in 167 in 2000 adults involved in a prenuptial examination (Gomez 2001).

It was also found that in a random sample of 1064 adults from Christchurch, New Zealand of whom 96% are Caucasians, 12 were positive for endomysial IgA antibody and one had been previously diagnosed with CD. Abnormal histology of mucosal small intestinal biopsies was confirmed in all patients with positive serology, which contributed to an overall prevalence of 1:82 or 1.2% (Cook 2000). In Australia, the overall prevalence of CD was estimated to be 1:251 or 0.4% of the population (Hovell 2001).

The disease has been well documented in Asians from India, Pakistan, and Iran but it is rare or nonexistent among native Africans, Japanese, and Chinese. The overall prevalence of CD in India is unclear due to limited data. CD was previously diagnosed more frequently in children than in adults, although this may reflect the acceptance of ill-health by some adults or lack of awareness of atypical manifestations (Cummins and Roberts-Thomson 2009). The majority of data originate from Northern India where wheat is the staple food rather than

rice that is more popular among southern population. In a field study conducted among school children in Punjab, the Northern Western part of India revealed the estimated frequency of disease was 1 in 310 (0.3%) (Sood 2006). This prevalence is probably an underestimation and there is a need to indicate studies to estimate the prevalence of CD throughout India (Gupta 2009).

The prevalence of CD in the Middle East is similar to that of Europe, Australia and New Zealand. CD is a relatively common in Iran, Israel and Egypt where wheat consumption ranks among the highest in the world. The prevalence rates for CD in Iran, Israel, Egypt and Turkey were 0.88% (Imanzadeh 2005), 1.1% (Israeli 2010), 0.53% (Abu-Zekry 2008) and 0.47% (Dalgic 2011) respectively. These prevalence rates are almost identical to those of a variety of countries in Europe. There are few clinical reports of the condition published in China and Japan. In these countries, the majority of the population consume rice as the staple food. Furthermore, human leukocyte antigen (HLA)-DQ2, a molecule that binds and presents peptide to antigen-specific T cells is virtually absent from the Japanese population but is present at low frequency in the Chinese population. Some new CD cases in China have been derived by the high frequency of HLA-DQ2 and HLA-DQ8 haplotypes within this population. In addition, individuals and common agricultural practices have resulted increased gluten consumption (Wu 2010).

1.3 Presentation of CD

1.3.1 Terminology and associations

CD has a great spectrum of clinical manifestations; this varies significantly between childhood and adulthood presentation. The CD predisposition is thought

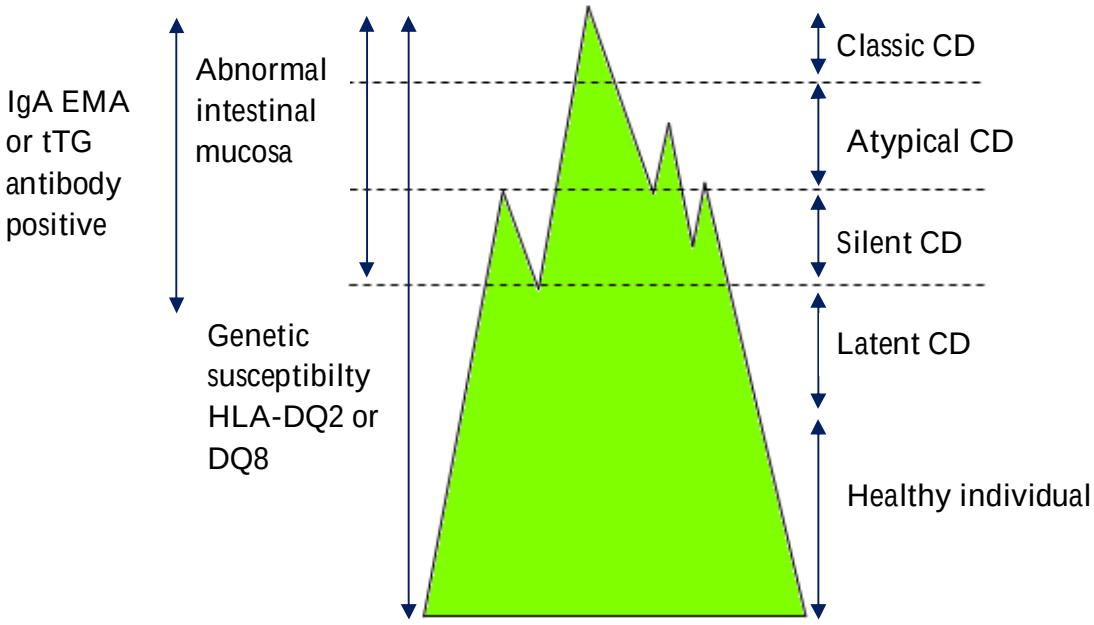
to start in early life as studies have found equivalent rates of positive serology in children as in adult populations (Maki 2003). There has been variability in terminology used to describe the presentation of CD; this leads to the difficulty when comparing and evaluating CD cases. A most recent manuscript has suggested definition and description for each CD term (Ludwigson 2013), although it is particularly intended for the Oslo population. The hidden or undiagnosed cases of CD are illustrated as Iceberg model, shown in Figure 1.3.

Classical CD was described as malabsorption, involving diarrhoea, steatorrhoea, weight loss and growth failure (Green 2005). These were however, no signs of malabsorption in non-classical CD (Ludviggson 2013). The most common non-classical manifestations of CD in children include iron deficiency anemia, short stature, and delayed onset of puberty. The term silent are referring to asymptomatic or CD without symptoms. These patients are said to have a "silent" form of the disease, they have positive coeliac serology but do not do not manifest the symptoms commonly associated with CD; they have no symptoms that respond to gluten withdrawal. Anemia is considered to be an atypical or silent presentation of CD, compared with the classic presentation with diarrhoea (Abu Daya 2013). Symptomatic CD is characterised by clinically evident gastro-intestinal and/or extra-intestinal symptoms attributable to gluten intake (Ludviggson 2013). Latent CD patients are found to have positive serology but a normal bowel biopsy even if they are consuming gluten. They may have had the symptoms in the past or develop them later in life.

The CD presentation is also linked to the typical condition that is the most frequently manifested among CD individuals such as malabsorptions, diarrhoea and weight loss. However, there are many other common manifestations that

can be classified as typical CD for instances; fatigue (Siniscalchi 2005), abdominal pain (van der Windt 2010), or dental enamel defects (Trotta 2013, Wierink 2007).

Figure 1.3: Coeliac iceberg re-illustrated from Rampertab and Mullin, 2014



1.3.2 Paediatric presentation of CD

The classical syndrome of diarrhoea has been previously known to be common among infants following the introduction foods possibly containing gluten (Vivas 2008, Telega 2008) but can also present in later life (Mearin 2007). Prolonged breastfeeding may postpone symptoms as the symptoms appear as early as the food introduction (Mäki 1988). Nowadays however, majority of children under two manifest diarrheal symptom (Rizkalla Reily 2012, McGowan 2009); whereas, older children and adolescent are reported to have abdominal pain, vomiting and constipation, and potentially other non-gastrointestinal manifestations such

arthritis, neurological disorder or anaemia (Tanpowpong 2012). The other manifestations in older children are short stature, delayed puberty, unexplained anaemia or iron-deficiency anaemia unresponsive to treatment, dental enamel defects involving secondary dentition, recurrent aphthous stomatitis, raised serum transaminase levels and osteopaenia. DH is a skin manifestation of CD; that improves with a GFD (Rodrigues and Jenkins 2006).

The most common symptom among paediatric CD in Scotland is abdominal pain (White 2013). Severe malnutrition and nutritional deficiencies, including rickets, may occur if the diagnosis is delayed. Behavioral changes such as irritability or lethargy are common. Coeliac-crisis which is rare can be also accompanied by ecchymosis (bleeding skin), hypocalcamic tetany, hypoalbuminemia, and oedema (Mearin 2007). Low bone density is also commonly seen among children with CD (Kalayci 2001) at the time of diagnosis although this is not a typical symptom. This in fact could become a precaution in preventing osteoporosis amongst adult CD individuals.

1.3.3 Adults presentation of CD

Diarrhoea presentation is the mostly common among adults, accounting for around 49% of CD individuals (Mukherjee 2008) but significantly lesser compared to approximately 100% of patients in the 1960s (Murray 2003). Weight loss, bloating, anorexia or abdominal pain, are mainly observed symptoms probably due to malabsorption. However, it has been reported that at least 30% of patients are overweight at the time of diagnosis (Lo et al. 2003). Besides symptom related to the gastrointestinal, the other presentations in adults include osteoporosis (Meyer 2001), iron-deficiency anaemia (Rodrigo-Saez 2011) and DH, a 'CD of the skin,' with a high frequency in CD adults (Hervonen 2002). In

adults, CD has been considered a pre-malignant condition, which can progress to lymphoma. Increased frequency for lymphoma (6%), small bowel adenocarcinomas, and oesophageal and oropharyngeal squamous carcinomas have been described. There is a form of malignancy, the enteropathy-associated T-cell lymphoma (EATL), with a very high association with CD; this is in general a rare condition with an absolute risk of only 1:1000 (cases of CD) based on the local prevalence of CD. Small bowel lymphoma and EATL are very rare diseases, but CD is the most important risk factor for these conditions. Bone density improves after introduction of a GFD, but in adult CD, this improvement generally does not reach the normal sex- and age-matched values for the control population. In contrast, in childhood CD with a very early treatment, gluten exclusion prevents bone loss and most children reach a normal bone mass.

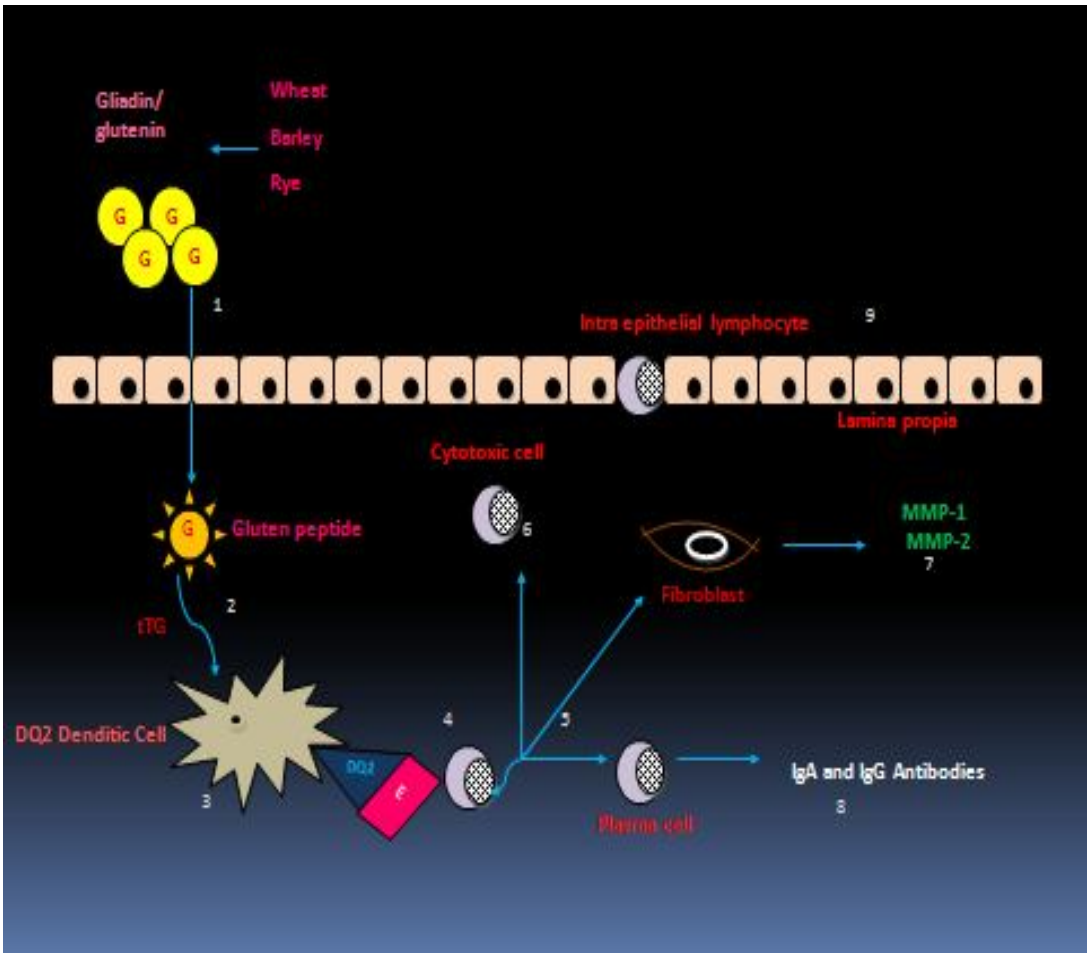
1.4 Pathogenesis

1.4.1 Genetic susceptibility

The genetic susceptibility of CD is influenced by the molecules in the HLA region. CD specific HLA-DQ2 and HLA-DQ8-molecules are expressed on antigen presenting cells (APCs); they present gluten peptides efficiently to T cell receptors of gluten sensitive T cells (Sollid 2002, Gebe 2002). Approximately 90% of CD patients carry the HLA-DQ2 heterodimer encoded by the DQA1*05 and DQB1*02 genes, carried either in cis on the DR3-DQ2 haplotype or in trans where the α chain is encoded on the DR5-DQ7 haplotype on one chromosome and the β chain on the DR7-DQ2 haplotype on the other chromosome (Qiao 2009). There is a lesser extent of CD association is among HLA-DQ2 negative patients who carry DQ8 molecules encoded by DQA1*0301/DQB1*0302 (Qiao 2009). Individuals who are homozygous for HLA-DQ2 were reported to have five

fold increased risk for the development of CD compared to heterozygous carriers (Tjon 2010). Besides genetic predisposition, CD is initiated by an inappropriate immune response to gluten fractions present in Triticaea (wheat, barley and rye) prolamins. This is generally accepted involving two systems, the adaptive and the innate immune response. The illustrated Figure 1.4 presents the generally accepted pathogenesis of CD.

Figure 1.4: Pathogenesis of CD



1. Gluten is digested to yield peptides, which are transported into the mucosa and enterocytes.
2. Key glutamine residues are deamidated by tTG.

3. Epitope processing and presentation occurs with HLA-DQ2 by APCs.
4. Gluten-sensitive T cells recognize CD toxic epitopes and are stimulated. IFNγ is secreted.
5. LP lymphocytes proliferate and recruit additional cellular infiltrate.
6. CD8 T cells with cytotoxic markers increase in number in small intestinal epithelium.
7. Fibroblasts are activated that produce metalloproteinase to degrade the matrix of the small intestinal.
8. Plasma cells are secreted to produce disease-specific coeliac antibodies, to tTG and endomysium.
9. The IELs may be involved in the innate response to gluten protein.

1.4.2. Adaptive immune response

Digested gluten peptides bind to HLA-DQ2 or DQ8 molecules on dendritic APCs on entering the lamina propria (LP) of the small bowel (Zimmer 2010). The presence of gliadin fragments alone can induce maturation of dendritic cells to serve as APCs (Palova-Jelinkova 2005). Prior to binding, deamidation of peptides is assisted by tTG (section 1.4.2.2) that increases the affinity of the binding to HLA molecules (section 1.4.2.1). At this point, the adaptive immune response is triggered in response to deamidated gluten peptides. The peptides are presented to T helper (Th) cells. The activated gluten sensitive Th-1 cells secrete inflammatory mediators and stimulate other immune cells. Interferon gamma (INF-γ) is released in response to Th-1 mediated inflammation (Troncone et al. 1998). The Th-1 cell activation also converts cytotoxic T lymphocytes into lymphokine-activated killer cells to attack enterocytes (Meresse 2004). IFN-γ also stimulates fibroblasts to release matrix metalloproteinase (Daum 1999) which

degrades the extracellular matrix, and also stimulates disease-specific plasma cells to produce anti-tTG antibodies which are initially released into the LP (Sollid 1997) then to the blood stream. B cells can also mature to become IgA antibody producing plasma cells.

1.4.2.1 Interaction between HLA molecules peptide binding grove and epitope

The peptide-binding properties of DQ2 have been extensively studied. Peptides which contain negatively charged anchor residues bind at relative positions P4, P6 and P7 for DQ2 and P1, P4, or P9 for DQ8 (Arentz-Hansen 2000). The preference for negatively charged residues for the three anchor positions in the middle seems to be unique for DQ2 (Figure 1.4.2.1).

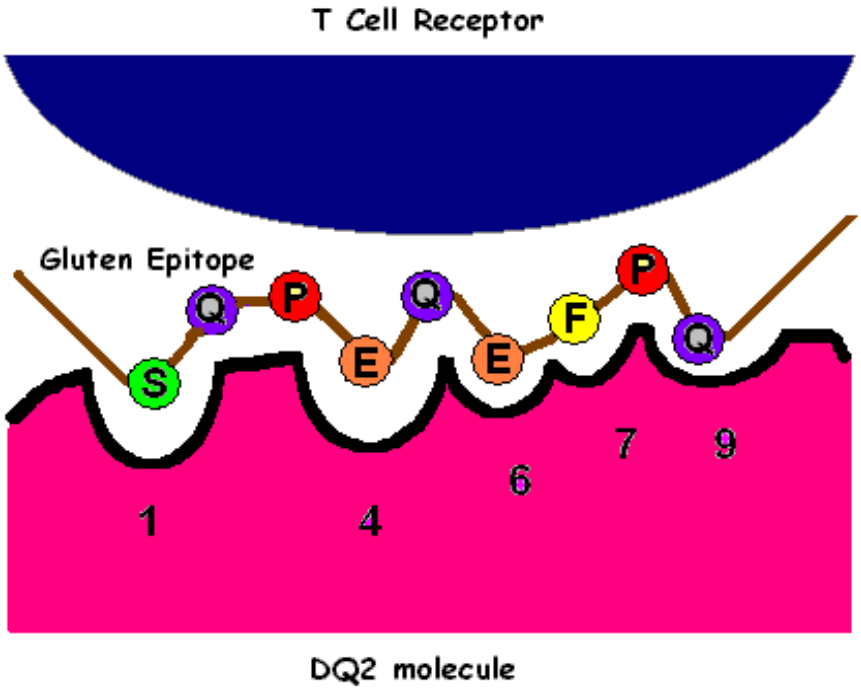


Figure 1.4.2.1: The diagrammatic representation of the interaction between DQ2 molecule peptide-binding grove and an epitope from γ-gliadin. Key anchor points are at positions 1, 4, 6, 7 and 9. Negative charge is preferred at positions 4, 6 and 7.

E represents deamidated residue with production of glutamic acid. **Q** and **P** represent glutamine and proline, respectively.

Gluten does not contain significant numbers of negatively charged residues but the charges can be introduced during post-translational modification by specific and targeted deamidation (Qiao 2009). Glutamine (Q) can modified to a glutamic acid (E) residue which has more negative polarity; this is catalysed by the enzyme tTG (Stenberg 2008) . The modified glutamine is accommodated in different pockets of DQ2 for the different epitopes. Any alteration within the peptide binding groove can lead to reduced reactivity, suggesting that T cell receptor interaction is critically sensitive to amino acid structure (Ellis 2003). Approximately 10-15 residues are required for optimal major histocompatibility complex (MHC) binding and T cell stimulation. For this, a peptic-tryptic (PT) or chymotropic digest of gluten may contain many suitable peptides (Dewar 2004) and can interact with the HLA binding grove.

1.4.2.2 Role of tissue transglutaminase

tTG is a ubiquitously expressed multifunctional protein and belongs to the vast transglutaminase enzyme family (Caputo et al. 2009). It is a calcium dependant enzyme that catalyzes the cross-linking of proteins by introducing a covalent bond between lysine (K) and glutamine (Q) (van de Wal, Kooy 1998). Its catalytic activity requires millimolar concentration of Ca2+ and it is inhibited by guanine nucleotides. Glutamine residues can be deamidated to glutamic acid as a side-reaction in the absence of suitable amines or at low pH (Caputo 2009). Patients with active CD have autoantibodies to tTG which are highly-specific and whose formation is dependent on ingestion of gluten (Burkhard 2004). The upregulation

of tTG in the damaged intestinal site will lead to the cross-linking of tTG to gliadin peptides or to other proteins resulting in the formation of additional antigenic epitope (Caputo 2009). tTG-gliadin peptide complexes act as hapten-carrier complexes, which could explain the typical antibody response against tTG in untreated CD patients (Burkhard 2004). tTG-gliadin complexes that bind to tTG-specific B cells, are endocytosed and processed. Gliadin-DQ2 complexes are then presented by the tTG specific B cells to gliadin-specific T cells (Caputo 2009).

The presentation and recognition of gliadin complex are necessary for the B cells to secrete tTG-specific antibodies (Burkhard 2004). It has been hypothesized that the production of anti-tTG antibodies is driven completely by intestinal gliadin-specific T cells, since tTG-specific T-cells seem not to exist (Caputo 2009). Autoantibodies against tTG are pathogenomonic of CD and identified gliadin epitopes contain at least one glutamine residue which is an excellent substrate for the deamidation activity of tTG (Mowat 2003).

It has been proposed that the native gluten peptide presented by HLA-DQ2 or HLA-DQ8 molecules to $CD4^+$ T-cells will lead the production of IFN-γ. IFN-γ results the HLA-DQ molecules to be highly expressed, therefore increases the presentation of gluten peptide. The increasing of gluten presentation causes minor tissue damage locally (Tjon 2010). tTG which is mostly intra-cellularly retained in an inactive form is activated upon its release during tissue damage either by mechanical stress, inflammation, infection or during apoptosis (Schuppan 2000). Deamidation by tTG increases the binding affinity of gliadin peptides to HLA-DQ molecules on the surface of APCs (van Belzen 2004) for T cells recognition (Molberg 1998). tTG enzyme converts the non-charged

glutamine into negatively charged glutamic acid (Tjon 2010). The deamidation of gluten is largely dependent on the presence of proline residues flanking the glutamine residues; gluten has a high content of these amino acids.

Motifs such as QXP, QXXF and QXPY represent the favoured target sites for glutamine deamidation whereas the sequence patterns, such as QP and QXXP inhibit the deamidation because of the presence of a proline residue (Vader 2003) The introduction of negative charges at positions favoured by HLA-DQ molecules significantly results in high affinity binding, thus increases the immunogenicity of deamidated peptides (Gebe 2002) and is essential for T cell stimulation (Mowat 2003).

1.4.3 Innate immune response

The innate activation of epithelial cells could be triggered along with adaptive immune response. Interleukin-15 (IL-15) is a pro-inflammatory cytokine involved in both adaptive and innate immunity. IL-15 released by APCs, is signalled by LP T cell IFN-γ (Waldmann 1999). In addition, LP APCs can produce IL-15 in response to specific gliadin peptide such as p31-43 (Maiuri 2003). This peptide has demonstrated CD toxicity in organ culture (Shidrawi 1995) and in-vivo (Sturgess 1994). Proliferation of cytotoxic CD8+ve intraepithelial lymphocytes (IELs) is promoted by IL-15 (Di Sabatino 2011).

IL-15 also stimulates IEL cytotoxic activity in duodenal epithelium via granzyme, perforin and Fas/FasL pathways (Junker 2009). The intestinal epithelial cells destruction could be pictured as the appearance of villous atrophy. It is not very clear which mechanism is predominant to the other, the adaptive or innate

response. The inflammatory effects are perhaps initialised by the epithelial activation then continuously trigger the LP response. The changes of pathological appearance in the mucosa of coeliac individuals have been noticed an hour after gluten ingestion (Maiuri 1996, Ciclitira 1984,) that could not be possibly caused by T cell activation.

1.5 CD exacerbation

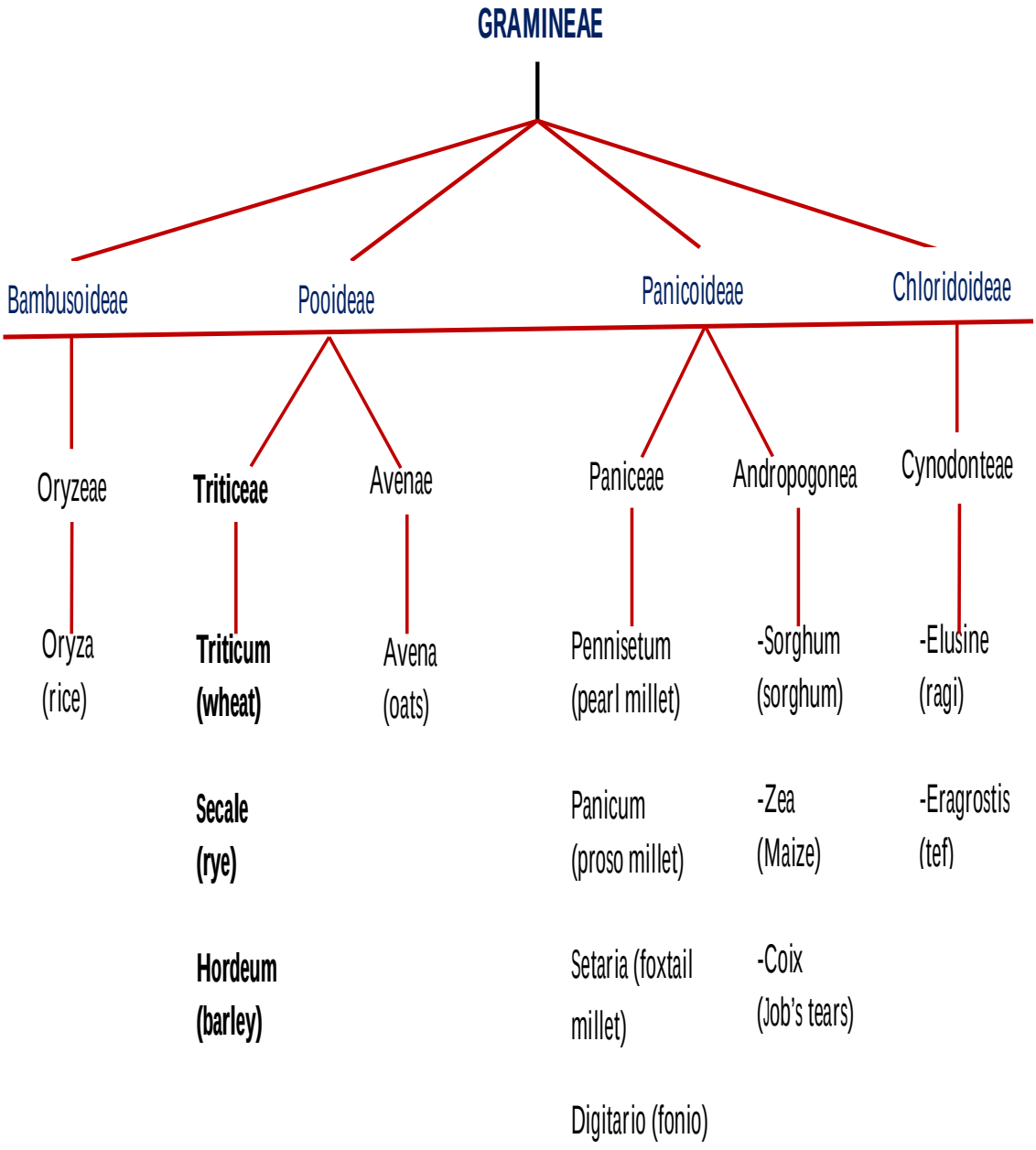
1.5.1 Taxonomy

In plant taxonomy, the grass family (Graminae) belongs to the plant kingdom subclass Monocotyledoneae or monocots. The only other grouping at the subclass level is that of Dicotyledoneae or dicots. Some species about which coeliac patients have questions are actually dicots, which have a very distant relationship to the grass family. Such species include buckwheat, amaranth, quinoa, and rapeseed. Because of their very distant relationship to the grass family including wheat, it is highly unlikely that these dicots contain the same type of protein sequence found in wheat proteins that causes problems for coeliac patients.

The three harmful species (wheat, rye, and barley) are members of the grass or Gramineae family and are quite closely related to one another according to taxonomy (Figure 1.5). However, not all members of the grass family damage the intestines of individuals with CD. Rice and corn (maize), for example, are apparently harmless. Members of the grass family that are more closely related to these species (based on taxonomy) than to wheat are likely to be safe. Such grasses include sorghum, millet, teff, ragi, and Job's tears, which appear to be closely related to maize. Species within the genus *Triticum* are almost certain to be harmful to individuals with CD. Grain proteins found in these species such as

spelt include the various types characteristic of the gluten proteins found in bread wheats (including the α-gliadins) that cause damage to the small intestine of coeliac patients. Rye (*Secale cereale*) and barley (*Hordeum vulgare*) which are toxic in coeliac patients belong to different genera, *Secale* and *Hordeum*, respectively, and retain certain common features to wheat.

Figure 1.5: The taxonomic relationship of cereals (confirmed toxic cereals are shown in boldface)



1.5.2 Prolamins

Gluten proteins are unusual in their high proline and glutamine content, comprising 15% and 35% of residues, respectively. A common feature of wheat prolamins is a high content of glutamine (>30%) and proline (15%), whereas the CD non-toxic prolamins of rice have a lower glutamine and proline content (Table 1.5.2). Proline is a secondary amide and causes a kink in the polypeptide backbone. Peptidases are unable to cleave the adjacent peptide bonds and therefore the presence of proline residues often dictates the fragments produced by proteolysis. Glutamine contains an additional γ-carboxamide group and can be deamidated to glutamic acid rendering a negative charge (Dewar 2004).

Table 1.5.2: The major prolamines that can drive the immune response in CD, which are rich in glutamine and proline (Schuppan 2000).

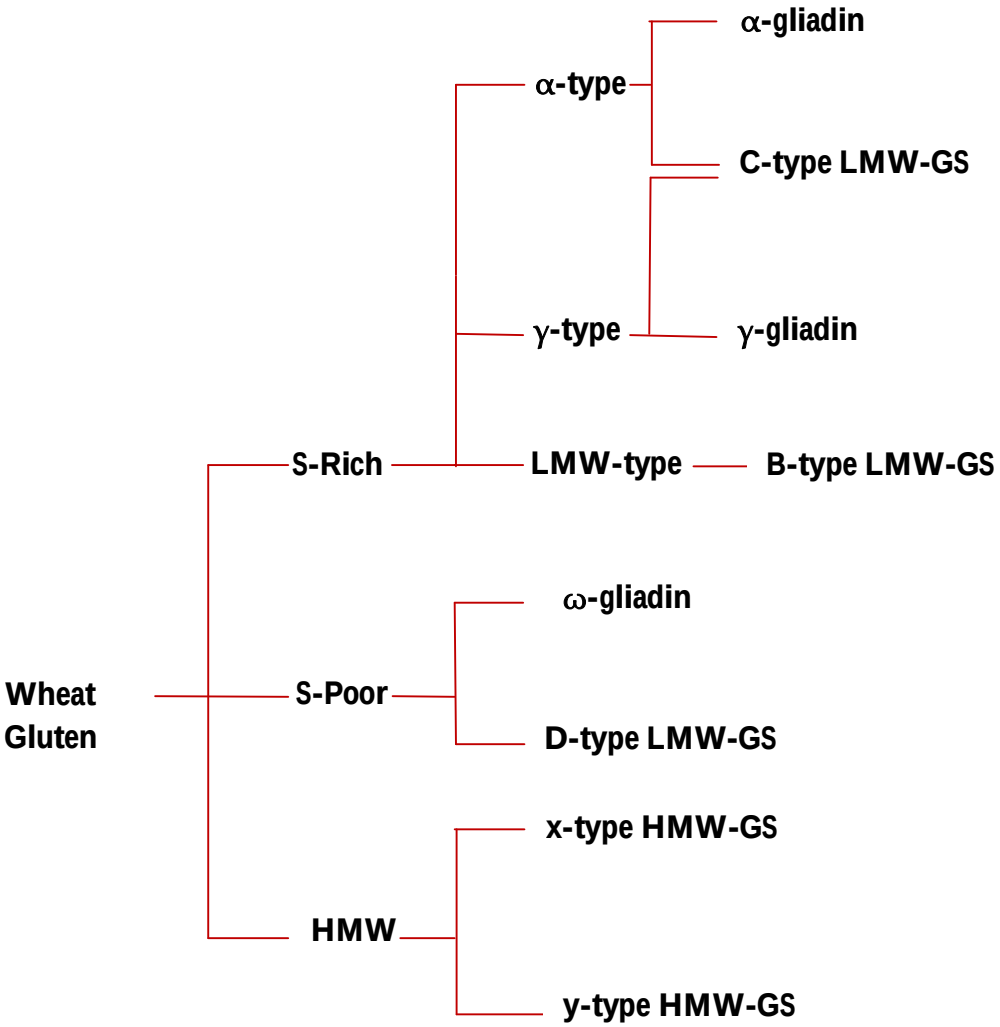
Cereal	Prolamin	Composition	
		Glutamine	Proline
Wheat	Gliadin	36%	17%-23%
Barley	Hordeins	36%	17%-23%
Rye	Secalins	36%	17%-23%
Oats	Avenins	High	Low
Maize	Zeins	Low	High
Rice	Rice prolamin	Low	High

1.5.2.1 Wheat gluten

Gluten is a rubbery mass that consists of storage proteins that remain after starch is washed from wheat-flour dough. These proteins have different solubility in alcohol/water solutions thus, can be roughly separated into two

fractions, gliadins (alcohol-soluble) and glutenins (alcohol-insoluble). Gluten proteins can be categorized into three main groups: 1) the sulphur (S) rich group (MW of 50kD), 2) the sulphur (S) poor group (MW of 50kD) and 3) the high molecular weight glutenin (HMW) with MW around 100kD. The first group comprises of ω-gliadin and D-type low molecular weight glutenin subunits (LMW-GS). The second consist of three major families: the B-type LMW-GS, the γ- and α-gliadins. Finally, the third group is further divided into two subgroups, the S-rich and S-poor (Figure 1.5.2.1). Gluten proteins have a complex chemistry and are responsible for the baking properties of wheat such as water absorption capacity, cohesivity, viscosity, and dough elasticity. The α-, γ-gliadins, B- and C-LMW glutenin subunits are grouped in S-rich prolamins. The S-poor prolamins consist of ω-gliadins and D-LMW glutenin subunits.

Figure 1.5.2.1: **The classification of wheat gluten proteins based on amino acid sequence (modified after Shewry and Lookhart, 2003)**



The alcohol soluble gliadins are predominantly monomeric proteins of 30-78 kDa which can be separated at low pH according to electrophoretic mobility in acid polyacrylamide gel electrophoresis (Shewry 1992). The separation results in four groups which are α-, β-, γ- and ω-gliadins. The α-, β-, and γ-gliadins contain intra-chain disulfide bonds which cannot be found in the ω-gliadins due to the absence of cysteine (Shewry 1992).

Gliadin has been mainly described as CD toxic (Mazzarella 2003, Fraser 2003, Ellis 2003, Anderson 2000, Arentz-Hansen 2000). In α/β gliadin molecules, several toxic epitopes were found such as gliadin-α2 (QPFPQPQLPYPQPQLPY) and gliadin-α9 (QLPPFPQPQLPYPQ) whereas, in the γ-gliadin molecule, gliadin-γ1 (PQQPQQSFPQQQRPF) and gliadin-γ2 (QQPFPQQPQQPFPQ) are the main toxic epitopes, which can present in hordeins (barley) and secalins (rye) (Vader 2003).

The alcohol insoluble glutenins can be divided into groups of HMW and LMW. Based on their mobilities on SDS-PAGE, three groups of LMW glutenins have been identified: B- (42-51kDa), C- (30-40kDa) and D-LMW (55-70kDa) (Lindsay and Skerritt 1999). HMW glutenin subunits are about 65-90kDa (Shewry and Halford 2002). Hydrated glutenins are responsible for dough strength and elasticity. Immunogenicity and toxicity in the HMW group have been well described (Ellis 2006, Dewar 2006, Molberg 2003, Vader 2002, Molberg 2001, van de Wal 1999).

1.5.2.2 Rye and Barley prolamins

Rye and barley have a unique composition of cereal prolamins due to the high content of glutamine and proline that is similar to wheat gluten. They are referred as secalin (from rye) and hordein (from barley), respectively. Both grains contain S-rich, S-poor and HMW prolamins, but no α-type S-rich prolamins. Two types of γ-type S-rich prolamins are found in rye; both are distinct in M_r and aggregation properties. Formation of polymers stabilised by interchain disulphide bonds are found in M_r 75 000 γ-secalin, whereas M_r 40 000 γ-secalin are monomers (Table 1.5.2.2). The homologous groups of S-rich S-poor and HMW prolamins present in rye and barley are summarised in Table 1.5.2.2 (Shewry 1992).

Table 1.5.2.2: Classification of rye and barley prolamins (Shewry 1992)

Components	Rye	Barley
S-rich prolamins		
γ-type	M_r 40 000 γ-secalin	γ-hordein
	M_r 75 000 γ-secalin	
α-type	-	-
Aggregated type	-	B hordein
S-poor prolamins	ω-secalins	C hordein
HMW prolamins	HMW secalins	D hordein

Besides wheat, they are known to be toxic to individuals with CD. They should be excluded from GF diet. Tye-Din (2010) has recently extended the evidence for immunostimulatory T cell epitopes in wheat, rye and barley. Apart from ω-gliadin peptide found to be the most immunodominant, Tye-Din has demonstrated other potential immunogenic peptides from barley and rye. His work has lead to the in-vitro immunogenic study that will be explained in Chapter Three.

Rye and barley have also previously been shown to be homologues to CD toxic gliadin (Vader 2003). Vader used T cell lines and clones from 8 different patients with CD to test 11 amino acid peptides from a selection of epitopes from rye and barley similar. They found that over 63% were recognised by gluten specific T cells lines or clones of CD patients. The gliadin-γ-2 epitope is not only present in γ-gliadin but also in secalins and hordeins. The gliadin-γ-2 homologues (present in secalins and hordeins) are reported to be equally recognized by gliadin-γ-2 specific T-cells. The secalin-γ-1 sequence also aligns at the same region of gliadin-γ-1, whereas, gliadin-α-2 and gliadin-α-9 homologues are present found in a secalin molecule (Vader 2003).

The immunogenicity of barley and rye prolamins was also investigated by quantifying the pro-inflammatory cytokines, IFN-γ and interleukin-2 (IL-2) using Taqman Polymerase chain reaction (PCR) and ELISA (Bracken 2005). It was shown that hordeins, and secalins resulted in significant increase of interferon γ-mRNA in small intestinal biopsies from individuals with CD.

1.5.2.3 Oat avenin

The status of oats has been reassessed in the international GF standard (Codex Alimentarius Commission 2008). They remain in the category of gluten containing cereals but there is an additional note stated that the use of oats not contaminated with wheat, rye or barley in foods is covered by the standard which may be determined on a national level (Codex Alimentarius Commission, 2008). Many extensive, long-term intervention studies with moderate intake of oats as part of GF diet have suggested its safety. The clinical study outcomes on oat challenge ranging from 2.5g of pure avenins to as much as 50g per day oat intake showed no histological changes (Huttner and Arendt 2010).

However, it was also indicated by the author that there were two publications (Arentz-Hansen 2004) and (Lundin 2003) that showed oats could induce mucosal inflammation in small subgroup of CD patients who are sensitive to oats (Huttner and Arendt 2010). Avenin sensitive T cell lines were obtained from small intestinal biopsies of individuals with CD. Oats were reported to improve the nutritional value of the GF diet without any negative effects on nutritional status. They therefore could help patients following a strict GFD (Butt 2008). It was also found that immunogenic sequences present in gliadin, are absent in oat avenins (Kilmartin 2003; Butt 2008).

Avenins contain half of the proline content found in other CD toxic prolamins including wheat gluten, secalins and hordeins (Huttner and Arendt 2010). In oats, protein classes are distributed differently with the largest group is dominated by globulins and only 10% representing prolamins. On the other hand, prolamins in wheat comprise about 80% of the total storage protein and 10% of the rest is globulin (Huttner and Arendt 2010). As such, oats are considered tolerable in individuals with CD.

The majority of oats available in Europe was reported to be contaminated with other CD toxic cereals. However, a study conducted with the synthetic avenin peptides demonstrated that the peptide epitope was not identical to the immunodominant gliadin peptide (PFPQPELPY). This suggests that there was no gliadin peptide contamination (Arentz-Hansen 2000). Many studies have seemingly shown that the intolerance to oats among CD patients is infrequent. Only a small number of patients have oat-sensitive small intestinal T-cells. Therefore, it is recommended that CD sufferers adhere to a strict GFD, as well as avoiding oats until clinical and histological remission has been achieved (Ellis and Ciclitira 2008). A recent study suggested that not all cultivars of quinoa are safe for CD patients to eat as evidenced by T cell activation (Zevallos 2012). However, the clinical feeding study implied that quinoa can be safely tolerated by individuals with CD (Zevallos 2014).

1.6 Diagnosis of CD

1.6.1 ESPGHAN guidelines for CD diagnosis

The European Society of Paediatric Gastroenterology and Nutrition (ESPGHAN) first proposed a guideline for CD diagnosis in 1970 (Meeuwisse 1970). The original statement for CD diagnosis is based on three criteria;

1) A minimum of three endoscopies based on structurally abnormal mucosa whilst still consuming gluten
2) Clear improvement of villous structure when commencing a GFD
3) Reappearance of mucosal damage resulting from gluten challenge.

In 1990, the criteria were revised (Walker-Smith 1990) after it was found gluten challenge were not required in all situations. The second and third biopsies were eliminated. Instead, the diagnosis should be based on the first biopsy and histological examination showing the characteristic abnormalities, that is, lymphocytic infiltration, villous atrophy and crypt hyperplasia, on a gluten-containing diet. In children under the age of 2 a gluten re-challenge and small intestinal biopsy is still required for the diagnosis of CD.

Based on ESPGHAN guidelines, an evidence-based approach (Husby 2012), three antibodies are considered as coeliac-specific. They are:

1) Endomysial antibody (EMA)
2) Transglutaminase 2-specific antibody (anti-TG2)
3) Deamidated gliadin-specific antibody (anti-DGP)

It has also been proposed that the histological assessment could be excluded if the individual show symptoms that can be referred to CD, the IgA anti-tTG levels is ten times higher than the upper normal limit, verified by EMA positivity and the individual is HLA DQ2 and/or DQ8 positive (Husby 2012).

1.6.2 Immunological markers and screening

Immunological response to the presence of gluten results in the production of auto-antibodies that is one of the characteristic of CD. Serological screening is the first crucial step for the CD diagnosis and is useful in monitoring GFD

commitment. The screening of CD has been improved by reliable serological markers; initially with IgA and IgG AGA, later the EMA and IgA anti-tTG antibodies, and most recently, deamidated gliadin peptide (DGP) antibody (IgA anti-DGP). The commonly used serologies are summarised in Table 1.6.2

Table 1.6.2: Comparison of serological tests for their sensitivity and specificity (modified after Leffler 2010)

Serological test	Sensitivity (%)	Specificity (%)	Positive predictive value (%),pretest probability of 5%	Negative predictive value (%),pretest probability of 5%
IgG AGA	85	90	18	99
IgA AGA	85	80	31	99
EMA	95	99	83	99
IgA anti-tTG	98	98	72	99
IgG anti-tTG	70	95	42	99
IgA anti-DGP	88	95	44	99
IgA anti-DGP	80	98	68	99
IgA/IGg anti-DGP	97	95	51	99

The first serological test was AGA developed in the early 1980s (Leffler 2010) that measured both IgG and IgA AGA. However, these antibodies were reported to have relatively low sensitivity and specificity that is between 80% to 90% (Naiyer 2009). AGAs were also reported to yield false-positives in patients with other causes of mucosal damage in the gastrointestinal tract. Much improved accuracy was reported with deamidated peptide AGA (Leeds et al. 2008).

The EMA testing was first described in CD more than 30 years ago and rapidly replaced AGA because of their higher specificity. AGA can be found in healthy individuals but EMA is unlikely to be found in non-CD patients. The test initially utilized monkey oesophageal tissue as substrate but was replaced later by human umbilical cord which was more sensitive and specific (McGough and Cummings 2005). The test also depended on immunofluorescence, and uses tTG as the target antigen (Leffler 2010). The sensitivity of IgA anti-EMA is generally more than 90% with a specificity of 97-100% (Fasano 2012). Despite of its high sensititivity, IgA anti-EMA is costly and inapplicable to evaluate CD individuals with IgA deficiency (Cataldo 1998, Salmi 2006). EMA was also found to be present in active CD but could be disappeared due to continuous GFD.

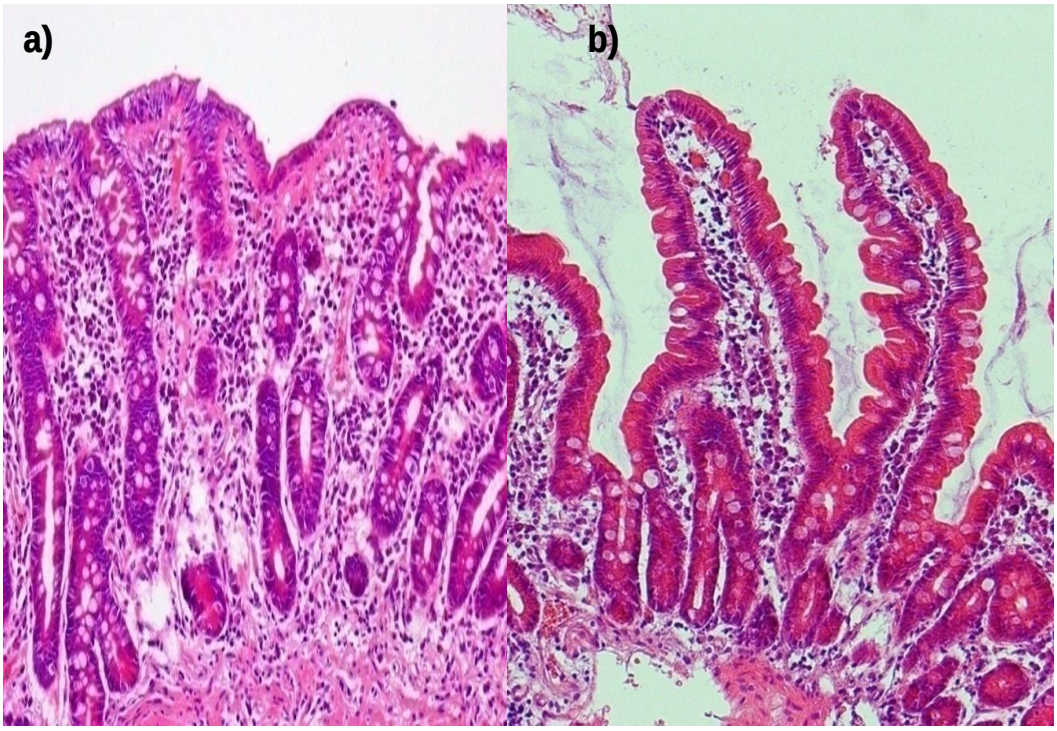
Following the recognition of tTG in the late 1990s (Dieterich 1998), IgA anti-tTG ELISA was developed. The first generation assays utilised guinea pig liver, subsequently replaced by human erythrocytes and human recombinant tTG (Dieterich 1998). The latter was reported to yield higher specificity when used as coating antigen (Lewis 2006, Tonutti 2003,). The anti tTG ELISA is simpler, less expensive and time saving (Van Meensel 2004). Hence, the IgA anti-tTG is recommended as the initial evaluation of CD. The IgG anti-tTG is also available for use in individuals with IgA deficiency (Fasano 2012).

Antibodies to DGP have been shown to be of diagnostic value (Volta 2010). The evaluation is based on the antibody response that has higher specificity for CD than antibodies to native gluten (Prince 2006). The IgA and IgG anti DGP assays were reported to have higher sensitivity than anti-tTG IgA and AGA IgA in monitoring compliance with GFD in children with CD (Monzani 2011), but anti-tTG IgA remains useful for CD screening (Stern 2000).

1.6.3 The histological of biopsies

The histological assessment of duodenal biopsies is the gold standard in the diagnosis of CD (Rostom 2006) that is performed after positive serological testing. The diagnosis is usually made on the basis of an abnormal duodenal biopsy on gluten containing diet and subsequent histological improvement after GFD (Figure 1.6.3).

Figure 1.6.3: Histological appearance of the stained small intestinal mucosa with magnification 100X, before and after GFD



a) Appearance of villous flattening, crypt depth hyperplasia and the expansion of LP with chronic inflammatory cells in the small intestinal mucosa. There are also increased numbers of lymphocytes within the epithelial layer.

b) Section of normal intestines with clear architectural presence of tall and slender villi, and normal crypt depth after GFD.

The histological assessments are described in Table 1.6.3 using Marsh-Oberhuber classification (Oberhuber 1999). The criteria observed on mucosal small bowel biopsy specimens include villous architecture, crypts changes and IEL counts/100 enterocytes (Marsh 1992, Oberhuber 1999). A Marsh I type comprises normal architecture of mucosa with lymphocytic filtration of the villous epithelial layer. Marsh II describes hyperplastic appearance with IELs in excess of 30 per 100 enterocytes. The villous height/crypt dept ratio will often become below the normal value 3-5. The particular feature of Marsh III is villous atrophy, that is extended to several degrees; the mild, moderate and total villous atrophy. A Marsh 4 describes a rare histological finding of a flat, atrophic mucosa thought to be resulted from chronic inflammation.

Table 1.6.3: Marsh-Oberhuber classification of CD

Marsh class	Type of lesion	Villous architecture	Crypts	IELs
Marsh I	Infiltrative	Normal	Normal	>30/100 enterocytes
Marsh II	Infiltrative-hyperplastic	Normal	Hyperplasia	>30/100 enterocytes
Marsh III				
3A	Flat destructive	Mild villous atrophy	Hyperplasia	>30/100 enterocytes
3B	Flat destructive	Moderate villous atrophy	Hyperplasia	>30/100 enterocytes
3C	Flat destructive	Total villous atrophy	Hyperplasia	>30/100 enterocytes
Marsh IV	Atrophic-hypoplastic	Total villous atrophy	Hyperplasia	>30/100 enterocytes

1.7 Complications and associations of CD

1.7.1 Autoimmune diseases

CD has been associated with several other conditions. Prolonged exposure to gluten may contribute to the development of autoimmune disease, or could be influenced by other factors (Sategna Guidetti 2010, Ventura 1999). CD presents atypically and unexpectedly in children and adolescents with type 1 diabetes mellitus (T1DM) (Goh 2007). It is considered the most notable complication that is around 11% of CD sufferers (Keskin 2010). The compliance to GFD improves health and weight, and reduces the hypoglycaemic episodes in some cases (Hansen 2006). Autoimmune thyroid disease (ATD) is another recognised association. The prevalence of ATD is reported ranging from 2.3% to 7.8% in children and adolescent (Sattar 2011). Around 6.4% of patients were reported to have autoimmune hepatitis (AIH), those who showed positive serologies and histological appearance associated with CD (Nastasio 2013).

1.7.2 Neurological manifestation

Neurological manifestations have been reported more prominent in adult patients than in paediatrics. Depression is one the most common condition that could happen if the diagnosis is delayed and the diet is being restricted to GFD. Depressed CD sufferers are prone to sleep disorders (Zingone 2010) and in some cases it could be severe to the extent of committing suicide (Ludvigsson 2011). Prevalence of chronic headaches, hypotonia, learning disabilities or ADHD has been reported increasing (Zelnik 2004). Hadjivassiliou and colleagues reported the neurological disorders in the majority of these patients that were termed as ataxia or peripheral neuropathy (Hadjivassiliou 1996). Accumulation of gluten ataxia is probably due to the

association with HLA-DQ2 and DQ8, the presence of circulating Purkinje antibodies and the presence of anti-tTG antibody in the gut and brain (Hadjivassiliou 2008).

1.7.3 Pregnancy and CD

Infertility and poorer outcome of pregnancy have known to be a complication of CD (Freeman 2010, Ozgor 2010). Women are reported to suffer recurrent miscarriage, low birth weight infant and increased mortality children (Sher 1994). The younger female had shown to have lower fertility, but higher fertility when they are older when compared to general population controls (Tata 2005). Affected fathers could suffer sub-fertility and indirectly linked to the low birth weight infant compared to offspring of an unaffected father (Ludvigsson 2001).

1.7.4 Ulcerative Jejunitis

Ulcerative jejunitis is often associated with refractory CD in adults but it has been recently reported to present in children. Ulcerative jejunitis is another cause of non-responsive CD. It is a rare disorder characterised by the ulceration of the jejunum and ileum. Scarring often occurs, leading to structure formation with alternating areas of dilated small bowel. Ulcers can be of varying depth and can extend through the entire thickness of the bowel leading to perforation (Bayless 1970). Steroids are commonly used to treat the ulceration and a strict GFD is essential.

1.7.5 DH skin disorder

DH is a gluten sensitive skin disorder (Figure 1.7.5) characterised by an intense itching and papulo–vesicle eruption primarily affecting the elbows, knees and buttocks (Moazzem Hossain 2009). On histological evaluation the

majority of DH patients are found to have an abnormal jejunal mucosa, however only 10% of patients suffer from any kind of malabsorption. Diagnosis is established by the demonstration of IgA autoantibodies at the dermo-epidermal junction, by immunofluorescence binding to these immune deposits. Recently, anti-tTG 3 antibodies have been shown to produce these deposits (Zone 2011). Patients who are advised to commence GFD usually show significant improvement within 6-12 months. Patients are also prescribed 50-100mg/ daily of Dapsone at the initial stage of manifestation and minimum dose required to control the rash may be reduced.

Figure 1.7.5: DH manifestations on back and elbow

1.7.6 Malignancy

CD has been associated with malignancies. The risk of malignancies has been reported not to be significantly after the patients comply with a GFD (Olen 2011). Coeliac patients have an increased risk of developing cancer if diagnosis is delayed (Silano 2007). This is probably due to the prolonged period of dietary exposure to gluten (Carrao 2001). This risk is more relevant for the

intestine-specific cancers such as small bowel carcinoma and non Hodkin's lymphoma. It has been also noted that CD individuals have a higher risk of developing a small bowel adenocarcinoma compared to the general population (Green 2004, Rampertab 2003). Adenocarcinoma of the small intestine is a rare cancer, demonstrated to affect 13% of patients with CD.

The overall survival at 30 was 58% and was not affected by the presence of CD (Howdle 2003). EATL is another type of rare lymphoma associated with CD, affecting 0.5-1.0 per million of Western populations (Delabie 2011). The survival from EATL is estimated 8-10% at 5 years (Al-Toma 2007). EATL has a peak incidence in jejunum in the sixth decade and is associated with refractory gastrointestinal symptoms as well as non-gastrointestinal sites.

1.8 Treatment of CD

1.8.1 GFD

The basis of CD treatment is lifelong adherence to a GFD that is avoiding products from rye and barley. In the vast majority, this leads to resolution of symptoms and a reduction in long-term risks of the complications associated with this disease. It was also reported that half of the CD patients do not achieve histological improvement with a GFD (Tye-Din 2010). The new Codex Alimentarius Standard for gluten-free food has been adopted following the agreement at European level. The new composition and labelling of foodstuffs suitable for people intolerant to gluten has been applied since January 2012, after being amended in 2008. The food labelled gluten-free may not contain wheat, rye, barley or oats, and its gluten level must not exceed 20 mg/kg whereas foods with gluten content 21-100mg/kg should be labelled as low gluten food (Codex Alimentarius Commission 2008).

However, complying with GFD is difficult and decreases quality of life due to poor sensational characterisation. A strict GFD is low in dietary fibre and folate, niacin and vitamin B_{12} (Thompson 2005, Thompson 2000). Several problems arise in relation to compliance with a GFD. First, the harmful gluten may contaminate food during the preparatory and processing steps (Ellis 2008b, Hernando 2008). Second, there is social pressure, especially in young patients to consume gluten (Fabiani 2000). Next, gluten-free products are not widely available and usually more expensive (Singh 2011). A GFD also has a poor palatability (Lerner 2010). Many gluten-free products are made from purified wheat starch (Ellis 2008b) that could contain traces amount of gluten.

1.8.2 Nutrition replacement

CD sufferers could consume naturally gluten-free cereals such as rice, millet, maize, sorghum and quinoa. Quinoa, contains all essential amino acids and high in minerals and vitamins that could be the perfect alternative to wheat. The consumption 50 g of quinoa over 6 weeks can be well tolerated by coeliac patients and did not exacerbate the condition. There was a histological improvement and positive trend in serological parameters, particularly a mild hypocholesterolemic effect (Zevallos 2014). In addition to compliance to GFD, patients with a low bone mineral density (BMD) should avoid smoking and excessive alcohol intake, and take calcium and vitamin D supplements (BSG Guidelines 2007).

1.9 Methods for assessing the toxicity of gluten fractions and peptides

This section describes techniques involving human tissue that have been used previously to study coeliac toxic gluten fractions, and some are being used with this current study. The current toxicity testing in is further subdivided into *in vitro* methods and *in vivo* methods.

1.9.1 *In vitro*

1.9.1.1 Organ culture of small intestinal biopsy

Browning and Trier first described a method by culturing a jejunal biopsy for 48 hours using an in vitro organ culture system (Browning and Tier 1969). This was subsequently employed to investigate the changes in cultured biopsies from patients on normal and a GFD, and controls in the presence or absence of prolamins (Falchuk 1974). The test substances used were PT gliadin or casein and untreated α-gliadin, with culture medium as a control. The parameters used to assess the biopsies were refined to use mean surface-enterocyte cell height (ECH), IEL count and villous height: crypt depth ratio (Howdle 1984). This technique requires only milligram quantities of peptides and around six biopsies are frequently obtained to conduct adequate tests. This method has now been used by a number of other researchers in assessing the toxicity of gluten fractions (Mazzarella 2003, Martucci 2003, Biagi 1999, Beckett 1999, Shidrawi 1995, Howdle 1984).

1.9.1.2 Small intestinal T cell culture

This assay is used to assess gluten sensitive T cells proliferation in response to the candidate peptides or prolamins presented by inactivated APCs. The APCs are the irradiated (22 Gray) peripheral blood mononuclear cells (PBMCs) from CD patients. T cells with phythohaemagglutinin (PHA) are used as a positive control. PHA is a plant lectin that triggers lymphocyte agglutination and proliferation. The T cells with APCs are used as the baseline for the stimulation indices (SI) calculation. Proliferation assays are undertaken when there is sufficient number of T cells. Cells are labelled for 18 hours with tritiated thymidine (^3H) before cell count is done. SI is calculated by dividing the mean counts per minute (CPM) of the T cells with APC and antigen by the mean CPM of the T cells with APC alone. Positive SI is noted as greater

than 2.0 and demonstrates the CD toxic of the tested antigen (peptides and prolamins) This method has been used to identify the toxic epitopes in gliadin by many researchers (Suligoj 2013, Zevallos 2011, Dewar 2006, Ellis 2003, Anderson 2000, Arentz-Hansen 2000) and applied in this current study. The adoption of this method is explained in details in Chapter Three.

1.9.1.3 Immunological tests of lymphocyte stimulation.

Peripheral blood lymphocytes are cultured in the presence of antigen that could stimulate the cell proliferation. Similar to the assessment of T cells proliferation (Section 1.9.1.2); the lymphocytes are cultured in the presence of radioactively labelled thymidine. DNA synthesis in cultured lymphocytes was reported to increase in the presence of Frazers Fraction III (Sikora et al. 1976). This method was also employed using PHA and Tuberculin purified protein derivative (PPD) as test antigens where the concentrations can be optimised. Previously, this was employed as the prelude of a test for cells proliferation before undertaking small intestinal *in vitro* study. The results are also expressed as stimulation index by dividing the average CPM of cells incubated with PHA or PPD with CPM of the cells (peripheral blood lymphocytes) themselves. The material used in this test is rather irrelevant to current CD studies compared to small intestinal T cell lines generated from CD biopsies.

1.9.2 *In vivo*

In vivo gluten challenge remains the gold standard method for testing gluten fractions and candidate peptides. Before any protein or peptide can be addressed as CD toxic or non-CD toxic to CD patients, feeding experiments will be performed before biopsies are obtained. In early study, gluten challenge was followed by faecal fat measurement (Van de Kamer 1953), however morphometric analyses of small intestinal biopsies have since been

taken into account. Treated coeliac volunteers undergo oesophagostroduodenoscopy (OGDs) at the beginning and at the end of the procedure. A Quinton hydraulic suction biopsy capsule is placed in the proximal jejunum by fluoroscopic control. The test peptide is infused intra-duodenally over an hour of time. Multiple biopsies are collected at hourly intervals following the gluten challenge. Morphometric assessment of biopsies is carried out under light microscope using these parameters: villous height/crypt depth ratio, epithelial surface ECH and IEL count. This method has demonstrated the dose-dependent effects on small intestinal biopsies and has been used to study potential toxicity of cereals peptides (Dewar 2006). It is an invasive method that requires a qualified physician and depends on the patients' compliance.

CHAPTER TWO: AIMS, OBJECTIVES, CONSIDERATIONS AND PLANS OF INVESTIGATIONS

2.1 Aims

The primary aim of this study was to investigate the coeliac immunogenicity of ω-gliadin/C-hordein peptide and rye secalin derived peptide with small intestinal gluten sensitive T cell lines. These peptides have been assessed with T cell clones of PMBCs and demonstrated high recognition to Triticae prolamins.

The secondary aim was to produce MAbs to these immunogenic peptides. This followed the demonstration of the stimulation in the majority of small intestinal gluten sensitive T cell lines when tested with these peptides.

The secondary aim was also sought developing an improved assay for gluten quantification in foods consumption by CD individuals. The development of ELISA in the cocktail competition format involved MAbs previously generated by investigators within our group. It is hoped that the combination of MAbs to both gliadin and glutenin, as well as MAbs to barley and rye fractions would yield an improved assessment of potential gluten contamination of commercially available foods for individuals with CD.

The null hypothesis of these studies is that both ω-gliadin/C-hordein peptide and rye secalin derived peptide are not immunogenic and do not stimulate small intestinal gluten sensitive T cells.

2.2 Objectives

The objectives are:

1. To investigate the coeliac immunogenicity from potential barley and rye peptides identified from literature as potentially toxic to individuals with CD. Following the potential CD toxicity of these peptides in PBMCs, these peptides were assessed with sensitive small intestinal T cell lines obtained from CD patients. We sought to generate wheat gluten sensitive T cell lines (n-14), barley hordein sensitive small intestinal T cell lines (n=9) and rye secalin sensitive small intestinal T cell lines (n=2).

2. To isolate PBMCs from CD patients' blood that would serve as APCs in the *in vitro* study.

3. To demonstrate the proliferation of sensitive small intestinal T cells in response to the presence of the potentially immunogenic peptides.

4. To develop MAbs to potentially CD toxic peptides.

5. To construct immunogens from the immunogenic peptides by conjugating to PPD.

6. To clone the resultant hybridoma for specific Mabs, followed by culturing and concentrating the tissue cultured supernatant containing the MAbs.

7. To characterise and evaluate the potential binding and cross reactivity of MAbs.

8. To design an improved assay for gluten quantification in commercially available foods for individuals with CD including purified wheat starch that serves as the basis for commercially available gluten-free food.

9. To establish cocktail competition ELISAs by combining available MAbs raised against to wheat glutenin and gliadin peptides.

10. To optimise the concentrations for coating antigen, competing antigen, and working antibodies using sandwich ELISA for the development of more sensitive and specific assay for gluten quantification in commercially available gluten-free foods.

2.3 Ethical considerations

The ethical committee approval was granted by the Ethical Committee of St Thomas' Hospital for the studies involving biopsies from CD patients. The LREC study number is 05/Q0702/167; the patients gave written informed consent.

The MAb procedures were approved by the United Kingdom Home Office and conducted at King's College London Biological Services Unit (BSU). The procedures were undertaken with the assistance from the supervisor under his Project Licence (70/19003) and Personal Animal Licence (70/6885). The animal work was classified as mild under the Home Office severity grading. The production of MAb is covered under the Animals (Scientific Procedures) Act 1986. All the procedures made to the mice were recorded and filed.

2.4 Confidentiality

All blood samples and tissues are anonymised by coding with the student's initial plus an alphanumeric code. All the information collected about patients involved in the study was treated as highly confidential and only documented on the individual consent forms. The forms are stored in as secure filing system and database to which access is strictly limited.

2.5 Plans of investigations

The overall strategy is shown in Figure 2.5.1 below:

1) Peptides of potential immunogenicity selected from literature, synthesis of peptides by solid phase peptide synthesis.

   a) ω-gliadin/ C-hordein peptide

   b) Rye-secalin derived peptide

2) Assess the peptides with gluten sensitive small intestinal T cell lines generated from CD biopsies.

3) Develop MAb to those peptides found to be immunogenic and CD toxic in the earlier *in vitro* study.

4) Develop an improved assay for quantification of gluten fractions if foods for CD individuals with greater sensitivity and specificity compared to those available.

CHAPTER THREE: INVESTIGATION OF CD IMMUNOGENECITY OF BARLEY AND RYE PEPTIDES

3.1 Introduction

CD is mediated by inappropriate recognition of, and activation of T cells to, immunogenic peptides of wheat, rye and barley. Numerous studies have demonstrated the presence of T-cell stimulatory peptides in gliadin (Vader et al 2002a, Vader et al 2002b, Anderson et al 2000, Arentz-Hansen et al 2000, van de Wal et al 1999, van de Wal et al 1998, and Sjostrom, 1998) and in glutenin (Tye-Din et al 2010, Dewar 2006 and Molberg et al 2003).

Barley and rye proteins have been known to be CD toxic, similar to wheat gluten, however little information is known regarding the immunogenic peptides within them. T cell responses to triticale proteins besides wheat gluten have been observed in CD patients (Tye-Din 2010, Kilmartin 2006 and Vader 2003), explaining the ability of barley and rye to be disease-inducing. Vader (2003) identified 11 homologous T cell stimulatory sequences in barley hordein and rye secalin, located in similar regions of the proteins. Seven of them were recognised by gluten specific T cell lines from CD patients.

Protein fractions of barley and rye was demonstrated to induce antigenic reactivity in gliadin sensitive T cell lines generated from CD small intestinal mucosa (Kilmartin 2006) such that these graminae need to be excluded from the diet of CD patients. Oats are considered safe in the majority of the CD patients (Ellis 2008, Janatuinen 2002), but occasionally not tolerated in 5% of CD patients (Lundin 2003).

Evidence suggests that most CD immunogenic peptides are found in α-gliadin. A protease-resistant 33 amino acid peptide is generally thought to be the most CD immunodominant antigen derived from α-gliadin that is recognised by gluten sensitive T cell lines from HLA-DQ2 positive CD subjects. In the light of Tye-Din and colleagues' study (2010), the evidence for potential epitopes in barley and rye has increased.

The first peptide candidate in this study was ω-gliadin/C-hordein peptide (**QPFPQPEQPFPW**). This potential immunogenic peptide was identified by Tye-Din and colleagues (2010), who tested with T cell clones of PBMCs, was shown to be the most stimulatory peptide in barley, wheat and rye, after the in vivo ingestion of these prolamins (Triticeae). T cell clones specific for this peptide were the most cross-reactive and recognised >50% of gliadin/hordein/secalin T-cell stimulatory peptides (Tye-Din 2010).

Perhaps the best explanation for the immunogenicity of this peptide is that the sequences are comprised of 2 overlapping CD-relevant T cell epitopes (Sollid 2012, Tye-Din 2010). The first epitope is DQ2.5-glia-ω1 (Sollid 2012) or known as DQ2- ω-I (Tye-Din 2010) which consists of 9 amino acids; **PFPQPEQPF**. The second epitope which also consists of 9 AA (**PQPEQPFPW**) is known as DQ2.5-glia-ω2 (Sollid 2012) or DQ2- ω-II (Tye-Din 2010). Vader (2003) considered DQ2- ω-I as a functional homolog of DQ2-α-1 (Hor-α-9/ Sec- α-9), and a similar view with DQ2- ω-2, being the functional homolog of DQ2-α-2 (Hor-α-2/ Sec-α-2). He pointed out that the toxicity of barley hordeins and rye secalins is due to the presence of Hor-α-9/ Sec- α-9 and Hor-α-2/ Sec- α-2 that were recognized by T-cells specific to DQ2-α-1 and DQ2-α-2. In contradiction to Vader's attribution, Tye-Din's work demonstrated overlapping DQ2- ω-I and DQ2- ω-2 as being

responsible for the toxicity of wheat, barley and rye, instead of DQ2-α-1 and DQ2-α-2 (Tye-Din et al 2010).

The understanding of the first peptide was further deepened by analysing its sequence using BLASTP (NCBI); the search yielded its closest homologies with C-hordein and ω-secalin (rye). This is in parallel with the study by Hsia and Anderson, (2001) that involved isolation and characterisation of ω-gliadin genes. Their analysis has confirmed the relation of wheat ω-gliadin to the C-hordein (barley) and ω-secalin (rye). This possibly supports the idea that this peptide exists in all three Triticeae prolamins, thought to exacerbate CD.

The second peptide candidate was considered from both Vader's (2003) and Tye-Din's (2010) studies. A rye derived peptide: **QPFPQPQQPIPQ** was recognized by more than 80% of PBMCs donors when assessed with IFN-γ enzyme-linked immunosorbent spot (ELISpot) following a five day oral gluten challenge (Tye-Din 2010). When one compares the sequence of this peptide with the aligned gliadin homologes in secalins (Vader 2003), it is shown that the second peptide lies in sequences of Sec- α-2 and Sec- α-9, thought to be homologue to DQ2-α-2 and DQ2-α-1, respectively. The peptide sequence was aligned using Constraint-based Multiple Alignment Tool (NCBI) and found that this rye derived peptide is a fraction of ω-secalin (sequence ID:gb|ACO40294.1| and ω-gliadin sequences (sequenceID: gb|ADF58080.1|).

The immunogenicity of the selected ω-gliadin/C-hordein peptide and rye derived peptide could be influenced by the proline (P) residue contents. These peptides can probably survive in the gut and remain, stimulating T cells. It has been reported that the proline-rich property protects gluten peptides from degradation

in the gastrointestinal tract so that they will persist (Shan 2002) and increase the chance they will bind to HLA-DQ molecules and trigger immune response. This could further explain the characteristics of ω-glaidin peptides being as immunodominant as α-gliadin peptides having high potential to bind to HLA, and trigger immune response.

In this chapter, the investigation CD immunogenic potential of candidate barley and rye peptides is described. This was sought by generating gluten-sensitive small intestinal T cell lines that were thought to be more pertinent to CD pathogenesis than PBMC lymphocytes. The focus in this study is the assessment of the peptides with small intestinal T cell lines sensitive to peptic-tryptic digested gluten (PTG).

In parallel to this, sensitive T cells were also raised to peptic-tryptic digested hordein (PTH) and peptic-tryptic digested secalin (PTS). Biopsies from patients were co-cultured with digested prolamins (peptic-tryptic) to sensitise the cells. Proliferation assays tested the responses to candidate peptides, and other Triticeae prolamins. The results of proliferations assays were analysed in terms of the CPM of incorporated tritiated thymidine, and stimulation indices were calculated. Sequential to this work, MAb to the immunogenic peptides will be raised with a view to developing improved methods for gluten measurement. This will be discussed in Chapter Four.

3.2 Aims

The primary aim is to investigate the potential CD immunogenicity of the ω-gliadin/C-hordein peptide and the rye derived peptide with gluten sensitive small

intestinal T cell lines established from biopsies obtained from individuals with CD. The secondary aim is to study the possible cross-reactivity of CD small intestinal gluten sensitive T cell lines with PTH and PTS.

3.3 Objectives

We wished to

- To obtain small intestine biopsies and blood samples from CD patients
- To isolate PBMCs from blood, freeze and use as APCs
- To establish wheat gluten small intestinal T cell lines
- To establish barley hordein and rye secalins T cell lines
- To undertake T cell proliferation assays to test the immunogenicity of the candidate peptides
- To study the cross reactivity between wheat gluten sensitive T cell lines with other cereal prolamins to confirm the presence of other shared peptides that could potentially toxic in CD patients.

3.4 Methodology

Gluten sensitive small intestinal T cell lines from patients were generated in order to undertake T cell proliferation assays. The established T cell lines were tested with the selected peptides, other Triticae prolamins and the co-culturing antigen itself, which is either PTG or PTH or PTS. PHA which serves as a marker for cellular viability was also included but the results were not shown.

3.4.1 Patients

Biopsies from CD patients were sensitised to PTG (n=14), PTH (n=9), and PTS (n=2). The patients used in this study were either treated with GFD or newly diagnosed and still consuming gluten. Ethical approval (05/Q0702/167) was obtained for all experiments and all patients involved had given informed consent before the procedures took place. Six to eight biopsies were obtained from CD patients. For the establishment of wheat gluten small intestinal T cell lines, the patients were coded with WG1, WG2 and so forth. Patients were coded as WB1 and WR1 for barley hordein T cell lines and rye secalin T cell lines, respectively. The ratio of female to male patients is 3:1. The list of patients whose biopsies were used to generate gluten sensitive T cell lines is shown in Table 3.1.

Table 3.1: List of patients recruited to generate gluten sensitive T cell lines

Code	Sex	Age (years)	Diagnosed (year)	GFD	Status
WG1 WB1	F	32	2011	4 months	DQ2
WG2 WB3	F	22	2007	5 years	DQ2
WG3 WB4	M	31	New patient	n/a	n/a
WG4 WB5	M	47	1997	15 years	DQ2.7
WG5 WR2	M	64	2012	4 months	DQ2
WG6	F	24	2011	4 months	DQ2
WG7 WB6	F	76	n/a	42 years	DQ2
WG8	F	29	2011	4 months	DQ2
WG9	M	50	2011	3 months	n/a
WG10	F	63	2011	1 year	n/a
WG11	F	30	2010	2 years	DQ2.8
WG12 WB7	F	35	2010	3 months	DQ2.8
WG13 WB8	F	65	2012	Eat gluten	
WG14 WB9	F	60	2011	10 months	DQ2.6
WB2 WR1	F	56	New patient	n/a	n/a

3.4.2 Peptides

The rationale for choosing the peptides in this study is explained in the introduction section (3.1). The synthetic peptides were designed with 16-AA residues, made commercially by GenScript (New Jersey, USA) with 95% purity. Candidate peptides are;

1. Omega (ω)-gliadin/C-hordein peptide, termed as **PepG**

   PQ**QPFPQPEQPFPW**QP

2. Rye-derived peptide, termed as **PepR**

   PQ**QPFPQPQQPIPQ**QP

3.4.3 Enzymatic digestion of gluten fractions-industrial gliadin

Wheat industrial gluten (418, Batch NW652) was purchased from Roquette Ltd, Corby Northants, UK). Pure milled flours of barley (variety Porter) and rye (variety Rheidol) were obtained from Plant Breeding International, Trumpington, UK. Triticae prolamins were prepared by pre-washing the milled flours with sodium phosphate buffer (0.4 M), to remove albumins and globulins, and extracting with 60% ethanol using a standard protocol (Wieser 1994).

The prolamins were enzymatically hydrolysed with pepsin (Sigma P0609) and trypsin (Sigma T1763), both attached to agarose according to the method of Bolte et al (1996), and others (Kilmartin 2003) with slight modification. One gram of wheat industrial gluten was dissolved in 50ml of 0.1mol/L hydrochloric acid (HCl), pH 2.0 and 105mg (4000U) of pepsin was added. The mixture was continuously stirred at 37°C for 2 hours and centrifuged for 20 minutes at 350 x g at 20°C. The pH was adjusted to 7.8 by adding 1mol/L of sodium hydroxide (NaOH); 1ml (25 U/ml) of trypsin suspension was then added. The mixture was continuously stirred and incubated for a further 2 hours at 37°C. The pH was then adjusted with 1mol/L HCl to 7.0. The suspension was again centrifuged for 20 minutes at 350 x g at 20°C. Finally the digested prolamin was dried by

evaporation at 37°C for several days and stored in a sterile bottle at room temperature for future use.

3.4.4 Antigen preparation

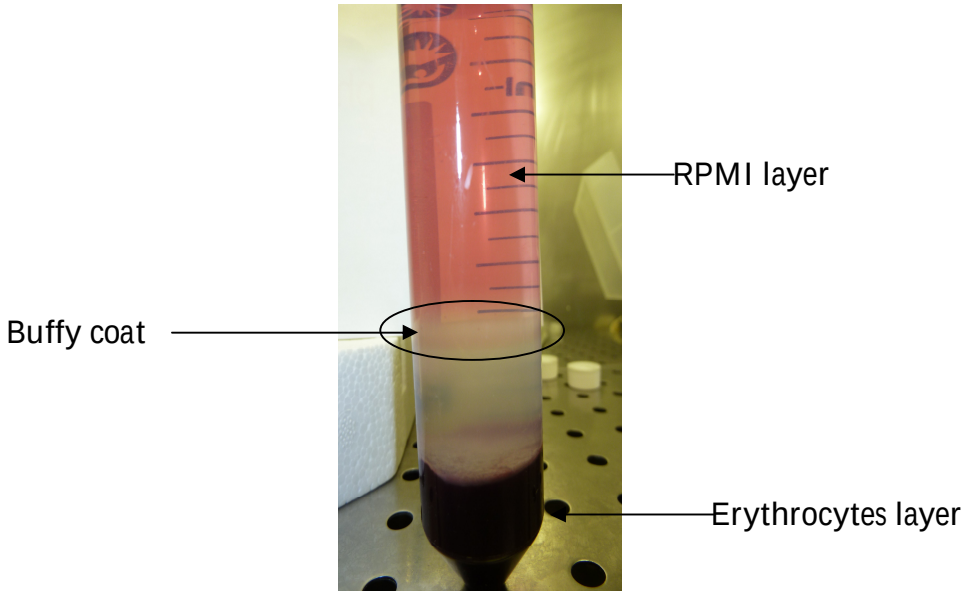
Deamidation of peptides is crucial for T cell recognition. In these T cell studies, peptic tryptic prolamins (PTG or PTH or PTS) were pre-treated with tTG to achieve deamidation. This was prior to incubation with APCs for use in T cell restimulation and T cell proliferation assays. Deamidated PTG was prepared by adding 10µl of PTG (10mg/ml) with 135µl of 2mM calcium chloride ($CaCl_2$) in PBS (Sigma, P4417) and 125µl of 200µg/ml tTG (Sigma, T5398) for four hours at 37°C. Following the incubation, the solution was irradiated for 30 minutes to sterilize and stored at -20°C. Similar steps were used to prepare deamidated PTH and PTS mixtures. The preparation of the stock solutions can be referred in Appendix 1

3.4.5 Isolating PBMCs from patients' blood

PBMCs were isolated to serve as APCs. The blood was collected from patients prior to endoscopy in a sterile syringe and transferred into 50ml Falcon tube(s) containing 100µl of heparin (1000U/ml, LEO V30814) to prevent the blood from coagulating. The blood was centrifuged at 800 x g for 10 minutes. The plasma supernatant was removed; 10ml of the remaining cell mixture was transferred to a 50ml Falcon tube. Twenty eight millilitres of Rosewell Park Memorial Institute (RPMI) medium (PAA) was added. This was mixed thoroughly, taking care to avoid producing bubbles. The mixture was then under- layered with 10ml of lymphoprep (Axis-Shield) and centrifuged at 800 x g for 20 minutes. The white cells contained in the 'buffy coat' (Figure 3.4.5), underlying the RPMI layer was

collected and transferred to a 50ml falcon tube. The white cell mixture was centrifuged at 350 x g for 15 minutes. The supernatant was carefully removed and the pellet resuspended in 5ml autologous serum medium (ASM) which contained 10% heat inactivated human serum, 25µg/ml plasmocin (InvivoGen), 2.5µg/ml amphotericin B (PAA) and 0.01M of HN-2-hydroxyethylpiperazine-N'2-ethane sulphonic acid (Hepes, Sigma H0887).

Figure 3.4.5: Separation of blood to obtain buffy coat



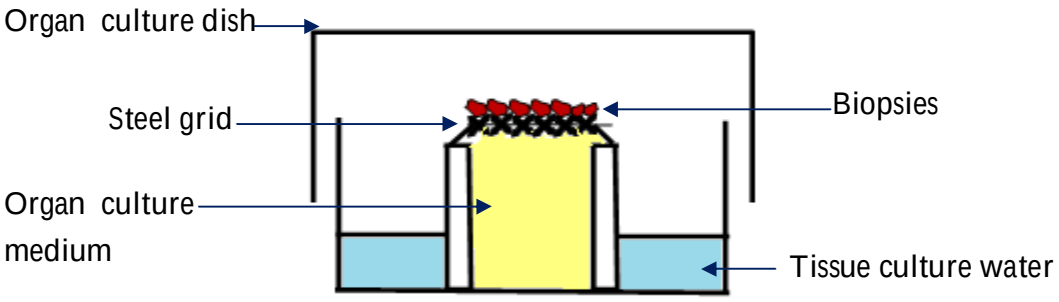
3.4.6 Obtaining autologous serum

Once the blood had been centrifuged, the clear upper layer that comprised the plasma was pipetted into a 15ml Falcon tube. The plasma was heated at 56°C in a water bath for 35 minutes to inactivate the complement proteins. The plasma was then centrifuged at 350 x g for 15 minutes. The plasma supernatant was collected into new tube and irradiated for 20 minutes (125 Gray) to be used in ASM. The tubes were stored at -20°C for future use within this study.

3.4.7 Isolating T cells from *in vitro* gluten challenged small intestinal biopsies

The establishment of small intestinal T cell lines in vitro was based on the methods of Ellis (2003). This method was sought to obtain T cells that could be cultured in a specific medium. Six to eight biopsies were obtained at endoscopy were placed in a single stainless steel grid on an organ culture plate. The sterile organ culture dish was prepared in advance by adding 1ml of filter-sterilised tissue culture water (Sigma W3500) to the outer well. The biopsies were oriented mucosal surface uppermost and close to each other to increase cell-cell contact (Figure 3.4.7).

Figure 3.4.7: Organ culture dish containing biopsies



The biopsies were cultured in organ culture medium containing PTG or PTH or PTS (5mg/ml) as the stimulating antigen. The central well was carefully filled with pre-warmed organ culture medium using a syringe, until a thin layer of medium was drawn over the serosal surface by capillary action. The organ culture dish was placed inside an organ tissue culture chamber.

A gas mixture of 95% O_2: 5% CO_2 was allowed to flow through the chamber for 1 minute prior to sealing the chamber. The chamber was then sealed and further gas was introduced until a pressure of 2 psi was reached. The cultures were incubated for 18-20 hours at 37°C in an atmosphere of 95% 0_2: 5% CO_2. The following day, after 18 hours incubation, the biopsies were harvested and homogenously dissected using two scalpel blades. The liquid beneath the grid was collected. The finely dissected biopsies were then passed through a 70µm nylon cell strainer (Falcon) which was placed on tube containing the organ culture liquid, to remove the epithelial cells and non-digested tissue. The debris remaining on the filter was retrieved and transferred into another 50ml Falcon tube. Into both tubes, ASM was added up to 10ml to re-suspend the cells that were then centrifuged at 170 x g for 7 minutes. The supernatant in each tube was discarded and the pellet was resuspended in 5ml of ASM. The tubes were centrifuged again at 170 x g for 7minutes.

The supernatant was discarded and the pellet resuspended in 2ml of ASM. Two x 10^6 irradiated (22 Gray) autologous PBMCs were added to the SIL fraction. The addition of PBMCs increased contact between the cells. Both suspensions were transferred in separate wells of a 24 well plate (Nunc), labelled as SIL and debris, respectively.

3.4.8 Freezing down cells

PBMCs were frozen to be used as APCs for restimulation of T cells. The suspension was frozen by adding drop-wise 20% ice-cold DMSO (Sigma D2650) in ASM, to an equal volume of cell suspension. The cells were aliqoted into several Nunc cryo-tubes, 2 to 5 x 10^6 in each tube. The cryo-tubes were placed in

cryo-chamber and frozen overnight at -70°C. The following day, the cryo-tubes were transferred to liquid nitrogen and left frozen for indefinite storage.

3.4.9 Maintenance of T-cell lines in culture

Interleukin-2 (IL-2) was added on day three and subsequently in three day intervals to a final concentration of 10U/ml. The addition of IL-2 stimulates the growth T cells that were sensitised due to the presence of gluten in the organ culture medium. Medium was refreshed every two or three days depending on cell growth rate whilst re-stimulation was undertaken every seventh day. The culture was re-stimulated with 2 to 5 x 10^6 of autologous, irradiated PBMCs which were incubated with the deamidated PTG (370µg/ml) for 18 hours before the re-stimulation. The cell line was subsequently maintained on a weekly re-stimulation cycle by feeding with IL-2 (to a final concentration of 10U/ml) every 3-4 days.

The growth of the culture was monitored by observing the colour of the medium. Rapid growth resulted in quick change of the medium red colour to yellow indicating the production of acid and the need for the culture medium to be refreshed. When cells were growing rapidly, cell suspensions were split by transferring either half or a third of the cell suspension to a new well. This was then made up to the volume of 2 ml with fresh ASM and 10% of IL-2 (200µl) was added.

3.4.10 Re-stimulation of culture with deamidated PTG

The cells were counted and checked for their viability prior to re-stimulation. If there were sufficient cells, proliferation assays could be undertaken. Otherwise,

cells would require re-stimulation with the antigen, that should be undertaken every seventh day. This interval would allow the cells to respond to the re-added antigen and proliferate. ASM, squeezing pipettes and 15ml Falcon tubes were placed on ice to cool as PBMCs were retrieved from liquid nitrogen storage. PBMCs were thawed under running warm tap water and immediately transferred to a pre-cooled Falcon tube.

Next, ASM was added to PBMC drop-wisely for the first 2ml then more rapidly up to a volume of 10ml. The mixture was centrifuged at 170 x g for 7 minutes. The supernatant was discarded and the pellet was resuspended with 10ml of plain ASM. The suspension was centrifuged again at the same condition to get rid of remaining DMSO. The pellet of PBMCs was resuspended in 500µl ASM and γ-irradiated (22-Gray). Irradiated PBMCs were used as APCs. The cells were transferred to a well (24-well culture plate). Five hundred microlitres (370 µg/ml) of deamidated PTG or PTH or PTS mixture was added and the resultant mixture was incubated at 37°C overnight. The PBMCs and antigen mixture was added to T cell cultures the next day to restimulate the T cells.

3.4.11 T-cell proliferation assay

T cells were assessed via T cell proliferation assays, in response to the presence deamidated PT prolamins and candidate peptides. PBMCs were thawed from liquid nitrogen storage to use as APCs. The PBMCs were counted using a haemocytometer. The number of cells required was determined by the number of wells used, allowing $5x10^4$ cells per well. PBMC were γ-irradiated (22 Gray) to prevent the APC themselves from proliferating. APCS were incubated at 37°C, 18

hours for more complex antigens including deamidated PTG/PTH/PTS and 4 hours for smaller peptides (PepG and PepR).

Deamidated PTG (or PTH or PTS) was used at a final concentration of 100μg/ml as they are more complex proteins while shorter sequences peptides were used at 10μg/ml to 20μg/ml due to their smaller size. The T cells and APC with PHA were included as positive controls at a final concentration of PHA of 10μg/ml as well as the T cells and APC alone as the baseline. Each test was established in triplicate. T cell lines for antigen reactivity were counted using a haematocytometer. A sufficient number of cells depended on the number of tests, allowing $5x10^4$ per well. The cells were transferred to a 15ml Falcon tube centrifuged at 170 x g for 7 minutes and resuspended in medium, 100μl per well. The suspension was added to APC pre-incubated with antigen, and incubation was carried out for 48 hours at 37°C.

One μCi/well of tritiated (^3H) thymidine was added in 20μl ASM to all the wells and the plate was incubated further for 18 hours at 37°C. Following the incubation, the cells were harvested using a Tomtec Cell Harvester. Tritiated thymidine incorporation into the nuclei of proliferating cells was measured using a Wallac 1450 Microbeta Plus Liquid Scintilation Counter (Perkin Elmer Life Sciences, Cambridge, UK). The SI for each antigen were calculated by dividing the mean CPM for the T-cells plus APC plus tested antigen by the CPM for T-cells plus APC alone, with PHA serving as a positive control for cell viability.

3.5 Results

Three categories of antigen sensitive small intestinal T cell lines were established to assess the immunogenicity of PepG and PepR. They were: 1) wheat gluten sensitive small intestinal T cell lines, 2) barley hordein sensitive small intestinal T cell lines, 3) rye secalin sensitive small intestinal T cell lines. CPM of small intestinal sensitive T cell lines in medium with and without antigen are shown in Table 3.2, Table 3.3 and Table 3.4. The SI were subsequently calculated by dividing the mean of CPM of triplicated samples of T cells + APCs + tested antigen (with antigen) by the values of T cells + APCs without antigen (blank).
SI > 2 is considered positive (Ellis 2003).

3.5.1 Statistical analyses

GraphPad Prism 5.0 for Windows was used to analyse the results statistically. As the number of samples is small and the data distribution is skewed (according to Gaussian distribution), non-parametric Freedman and Kruskal Wallis tests were employed followed by Dunn's multiple comparisons tests.

3.5.2 Generation of wheat gluten small intestinal T cell lines

Fourteen T cell lines sensitive to wheat gluten were generated. These lines were obtained from co-culturing the biopsies of CD patients for 18h in the presence of PTG (5mg/ml). The results were interpreted in two ways: one was by comparing the CPM, and the other, the comparison of SI for the selected peptides with PTG, which served as a positive control. The cells without added antigen formed the baseline. As shown in Table 3.2, the results are expressed as arithmetic mean of CPM ± SEM, the other column show the calculated SI. Tested antigen triggered intestinal responses in the majority of studied patients, and a heterogeneous

pattern was shown. Comparison of results with and without antigen is shown in Figure 3.1. Comparison of SI for each tested antigen is with PTG is shown in Figure 3.2.

The response of wheat gluten small intestinal T cell lines in each patient is illustrated in Figure 3.3. The line-graph of two T cell lines displayed higher peaks compared to the other T cell lines.

Table 3.2: Proliferation assays of wheat gluten (PTG) small intestinal T cell lines.

Patient no.	R (s)	T cells +APC **No antigen**	T cells +APC **PTG**		T cells +APC **PepG**		T cells +APC **PepR**		T cells +APC **PTH**		T cells +APC **PTS**	
		Mean CPM ±SEM	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI
WG1	2	98.35 ±22.79	820.25 ±62.47	8.34	641.75 ±135.05	6.52	853 ±146.55	8.67	478.1 ±113.66	4.86	495.33 ±127.15	5.03
WG2	2	213.67 ±11.29	625.33 ±86.67	2.93	465 ±33.05	2.17	314.7 ±29.49	1.47	282.67 ±16.74	1.32	601.33 ±309.95	2.81
WG3	2	312.525 ±34.88	2435.3 ±144.06	7.93	834.37 ±110.67	2.67	807.1 ±39.46	2.58	1412.67 ±127.41	4.52	1900 ±254.74	6.07
WG4	1	178.250 ±42.42	11157.5 ±998.59	62.6	22629.3 ±1755.52	127	16276 ±1824.97	91.3	13822.2 ±687.16	77.5	4128.33 ±259.81	23.2
WG5	1	335.625 ±74.81	3084.75 ±125.61	9.19	1945.08 ±121.77	5.79	2847.2 ±172.33	8.48	1608.4 ±141.15	4.79	2414.9 ±717.81	7.19
WG6	1	35.675 ±4.95	447.67 ±19.852	12.5	58.27 ±9.35	1.63	99.967 ±20.28	2.8	40.8 ±1.67	1.14	70.9 ±28.95	1.99
WG7	3	40.050 ±3.65	329.28 ±16.01	8.2	48.57 ±10.8	1.21	32 ±3.0	0.8	53.43 ±11.94	1.33	194.23 ±66.22	4.85
WG8	2	320.500 ±21.65	2345.87 ±57.16	7.32	483.67 ±32.57	1.5	461.67 ±8.212	1.44	1493.8 ±76.42	4.67	412.67 ±18.42	1.29

Tested antigens are PTG, ω-gliadin/C-hordein (PepG), rye-derived peptide (PepR), PTH and PTS. R(s) is number of restimulations, CPM is counts per minutes, SEM is standard error of mean and SI is stimulation index.

Patient no.	R (s)	T cells +APC **No antigen**	T cells +APC **PTG**		T cells +APC **PepG**		T cells +APC **PepR**		T cells +APC **PTH**		T cells +APC **PTS**	
		Mean CPM ±SEM	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI
WG9	2	214.675 ±16.39	7777.97 ±27.7	3.62	222.43 ±17.0	1.03	223.33 ±4.83	1.04	694.47 ±50.55	3.23	296.3 ±23.22	1.38
WG10	3	39.150 ±11.42	1198.67 ±62.73	30.6	2217 ±263.77	56.6	1502 ±20.27	38.4	501 ±100.98	12.9	596.67 ±58.08	15.2
WG11	3	136.000 ±29.82	1661.0 ±165.1	12.2	1821 ±438.44	13.4	1696.3 ±202.35	12.5	1231 ±136.9	9.05	2060.33 ±204.26	15.2
WG12	2	189.500 ±45.04	1280.0 ±132.29	6.75	1986 ±149.16	10.5	934.33 ±18.98	4.93	737.67 ±151.43	3.9	1302 ±149.51	6.87
WG13	3	40.800 ±9.379	416.6 ±48.82	10.2	140.83 ±27.88	3.45	101 ±31	2.48	134.07 ±36.43	3.29	0	0
WG14	3	31.100 ±6.8	80.75 ±9.39	2.6	51.47 ±8.5	1.65	53.4 ±2.59	1.72	54.3 ±4.84	1.75	58.27 ±6.73	1.87

Tested antigens are PTG, ω-gliadin/C-hordein (PepG), rye-derived peptide (PepR), PTH and PTS. R(s) is number of restimulations, CPM is counts per minutes, SEM is standard error of mean and SI is stimulation index.

Mean CPM of triplicate wells of T cell with and without antigen (blank: APCs+T-cells only) were compared for significance using a Friedman test (P<0.001). The Dunn's multiple comparison post test determined that, in relation to CPMs, all T cell lines with tested antigen (PTG, pepG, pepR, PTH and PTS) were significantly different from T cell lines without antigen (P<0.001, P<0.001, P<0.01, P<0.05, P<0.001), respectively. The spread of CPMs are represented in box plots (Figure 3.1).

Figure 3.1: CPM of wheat gluten T cell lines with and without the presence of tested antigen

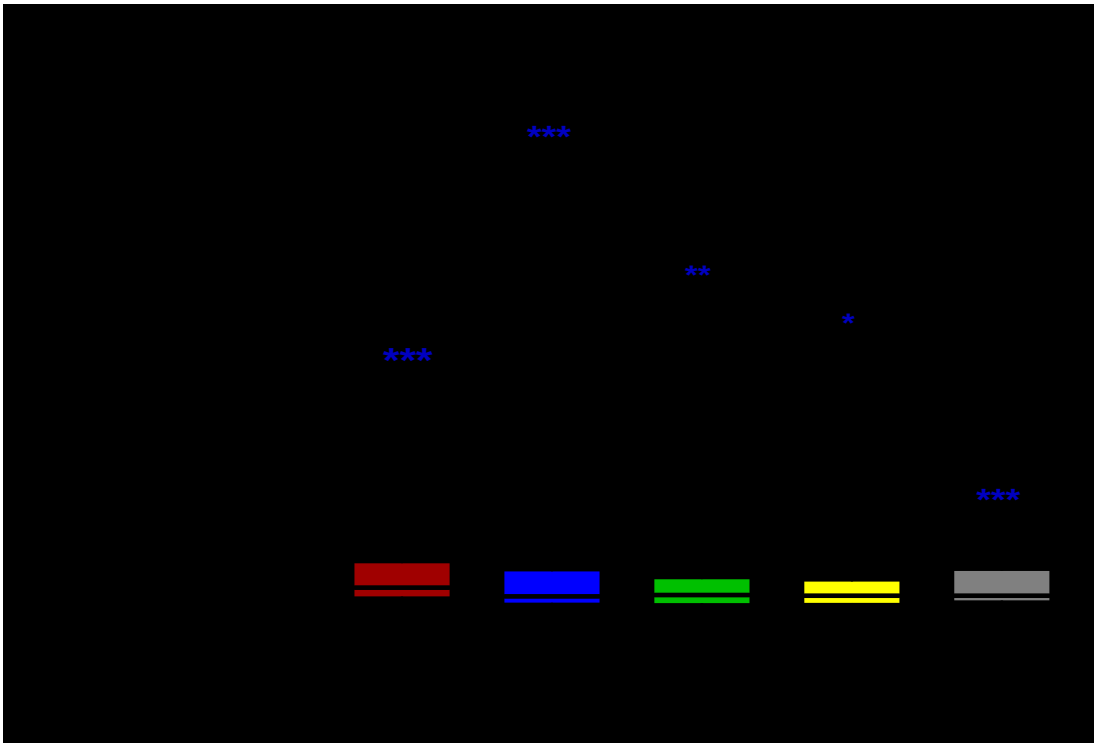


Figure 3.1: Bars are means of CPM; the error bar is standard error of means (SEM). The lines in the boxplots represent the median value. Significant difference are shown by * (P<0.05), ** (P<0.01) and *** (P<0.001). PTG is peptic tryptic gluten; PepG is ω-gliadin/C-hordein; peptide PepR is rye-derived peptide; PTH is peptic tryptic barley hordein and PTS is peptic tryptic rye secalin.

The median (SI) obtained from the all tested antigens were compared with the median (SI) of PTG (Figure 3.2). PTG served as positive control to demonstrate the specificity of the T cell lines that was used in the cultures in the beginning. S.I to PTG was between 2.6 to 62.6 (median=8.28). Strong responses to the ω-gliadin/C-hordein peptide and the rye–derived peptide were displayed in most of the gluten specific T cell lines, with SI varying between 2.17 to 126.95 (median = 3.06) and 2.48 to 91.31 (median = 2.69), respectively. There was cross-reactivity between wheat gluten sensitive T cell lines with PTH (n=10), the SI ranging from 3.28 to 77.54 (median= 4.2) and PTS (n=9), with SI of 2.8 to 23.16 (median = 5.03). Responses to the substrate PHA (data not shown) were high, indicating that the cells were proliferating in response to this mitogen. Kruskal Wallis test ($P<0.05$) with Dunn's multiple comparison post test determined that, the proliferative responses of both peptides were not significantly different from PTG, showing that stimulating effect of both peptides were as strong as the positive control, PTG.

Figure 3.2: Comparison of SI for each tested antigen

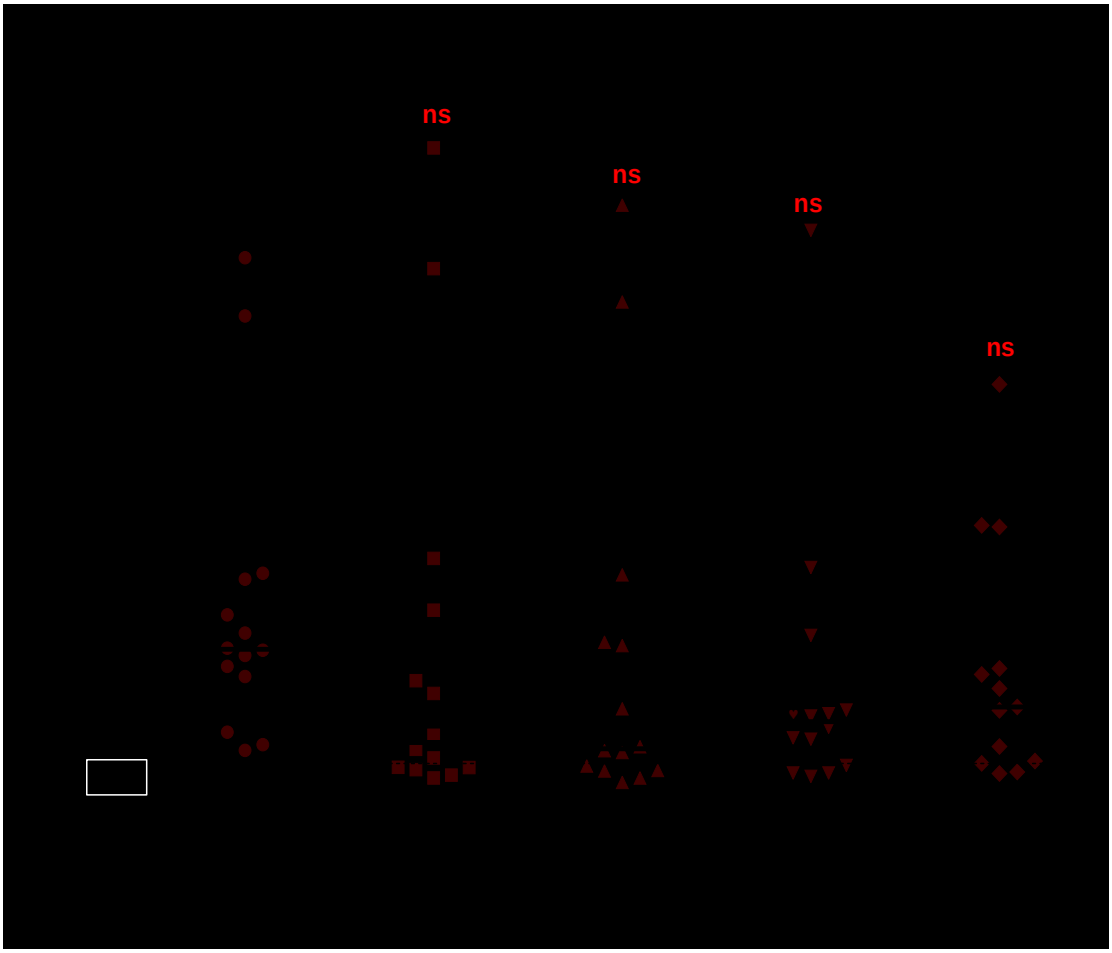


Figure 3.2 Each dot represents an SI for an individual assay. The lines represent the medians of the value for each tested antigen, **ns** indicates not significant, compared to PTG. PTG is peptic tryptic gluten; PepG is ω-gliadin/C-hordein; peptide PepR is rye-derived peptide (QPFPQPQQPIPQ); PTH is peptic tryptic barley hordein and PTS is peptic tryptic rye secalin

The response for individual coeliac patients is shown in Figure 3.3. The SI for WG4 and WG10 to all tested antigens are markedly higher than for all other T cell lines. The majority of T cell lines positively responded to all tested antigens (SI between 2.48 to 126.95). T cell lines of WG14 did not respond to all tested antigen except showing positivity reactivity to PTG. Negative responses were

demonstrated by T cell line of WG7, WG8 and WG9 to both peptides despite being reactive to antigen PTG.

Figure 3.3: The SI for individual patient to all tested antigens (wheat gluten sensitive small intestinal T cell lines)

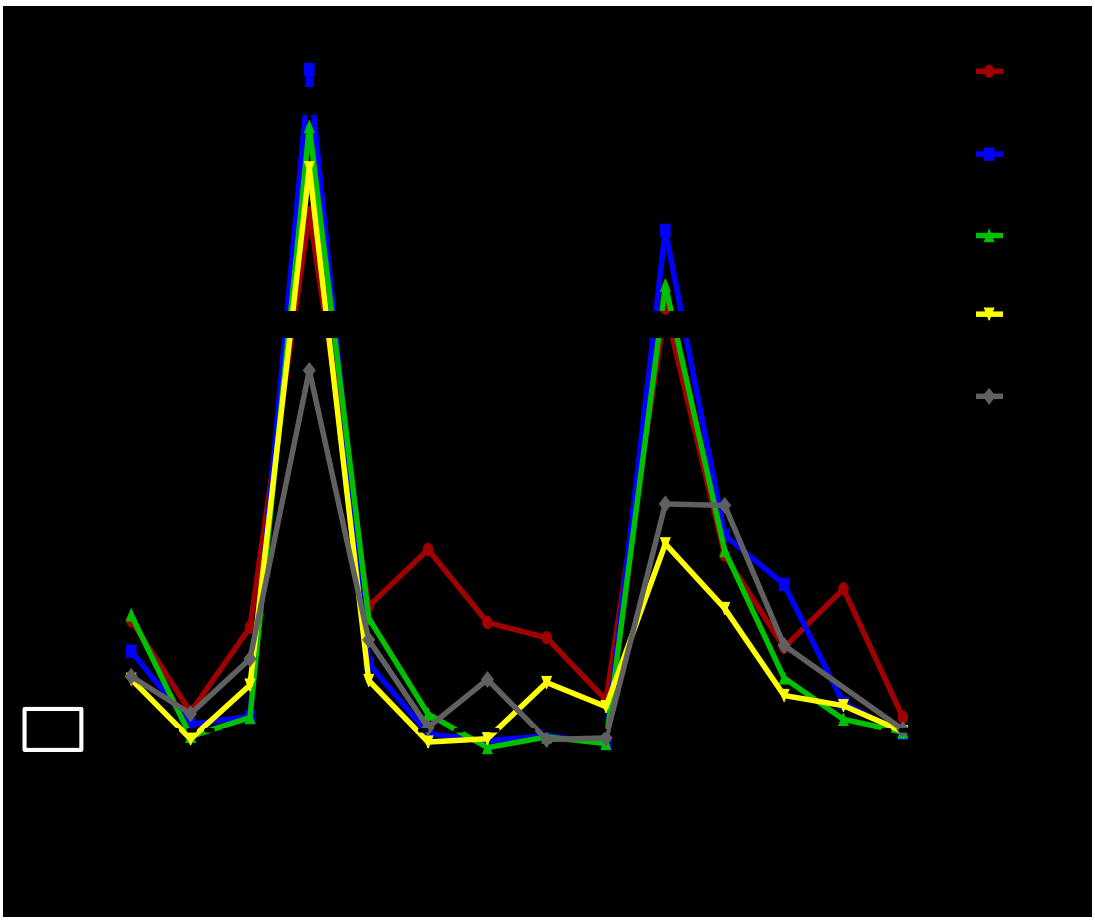


Figure 3.3 Each line corresponds to SI obtained per antigen tested with 14 T cell lines, labelled with WG1-14. A dotted line at SI of 2 distinguishes positive from negative results of proliferation assays. PTG, peptic tryptic gluten; PepG, ω-gliadin/C-hordein peptide; PepR, rye-derived peptide; PTH, peptic tryptic barley hordein; PTS, peptic tryptic rye secalin

3.5.3 Generation of barley hordein small intestinal T cell lines

Nine T cell lines sensitive to barley hordein were generated by co-culturing the biopsies of CD patients for 18h in the presence of PTH (5mg/ml) to sensitise the cells. The proliferative responses were analysed in term of mean CPM, and SI. PTH was the positive control, and blank (without the antigen) was the baseline. As shown in Table 3.3, the results are expressed as arithmetic mean of CPM ± SEM, the other column show the calculated SI. The tested antigens were shown to trigger heterogeneous intestinal responses in the majority of studied patients, particularly from both peptides.

One barley hordein sensitive T cell line has demonstrated noticeably high proliferative response to PTH (SI=12.43) and all tested antigens.

Table 3.3: Proliferation assays of barley hordein (PTH) small intestinal T cell line

Patient no.	R (s)	T cells +APC **No antigen**	T cells +APC **PTH**		T cells +APC **PepG**		T cells +APC **PepR**		T cells +APC **PTG**	
		Mean CPM ±SEM	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI
WB1	3	407 ±61.11	1739.5 ±227.37	4.28	2403.25 ±203.66	5.9	1698.75 ±202.95	4.17	917 ±283.89	2.25
WB2	2	95.167 ±21.02	268.067 ±27.55	2.81	139.83 ±25.46	1.47	194.23 ±8.65	2.04	366.17 ±17.00	3.85
WB3	3	395.38 ±26.71	2179 ±328.81	5.51	3101.67 ±326.84	7.84	2388.33 ±80.63	6.04	2926 ±142.7	7.4
WB4	3	141.95 ±25.69	1482.75 ±89.92	3	157.25 ±35.42	1.1	165.5 ±29.32	1.16	1908.25 ±163.7	13.4
WB5	1	679.5 ±49.42	8449.25 ±1200.17	12.4	20960.3 ±970.3	30.9	27665.8 ±410.83	40.7	40530.8 ±804.74	59.6
WB6	2	179 ±8.59	671.75 ±31.49	3.75	1442 ±46.57	8.05	1675 ±128.22	9.36	989.5 ±113.65	5.52
WB7	2	376.67 ±81.41	792.67 ±68.96	2.1	1045.33 ±39.06	2.77	1093.67 ±79.74	2.9	1373.67 ±197.33	3.64
WB8	2	115.57 ±10.12	311.77 ±47.1	2.7	410.83 ±144.26	3.55	426.4 ±55.68	3.69	1666.53 ±195.83	14.4
WB9	2	34.95 ±6.06	168.03 ±24.05	4.8	27.2 ±5.14	0.77	21.37 ±5.12	0.611	262.23 ±38.79	7.5

Tested antigens are PTG, ω-gliadin/C-hordein (PepG), rye-derived peptide (PepR), and PTH. R(s) is number of restimulations, CPM is counts per minutes, SEM is standard error of mean and SI is stimulation index.

Mean CPM of triplicate wells of T cell with and without antigen (blank+APCs+T cells only) were compared for significance using a Friedman test (P<0.001). The Dunn's multiple comparison post test determined that, in relation to CPMs, all T cell lines with tested antigen (PTH, pepG, pepR and PTG) were significantly different from T cell lines without antigen (P<0.05, P<0.05, P<0.05, and P<0.001, respectively). The spread of CPMs are represented in box plots (Figure 3.4).

Figure 3.4: CPM of barley hordein T cell lines with and without the presence of tested antigen

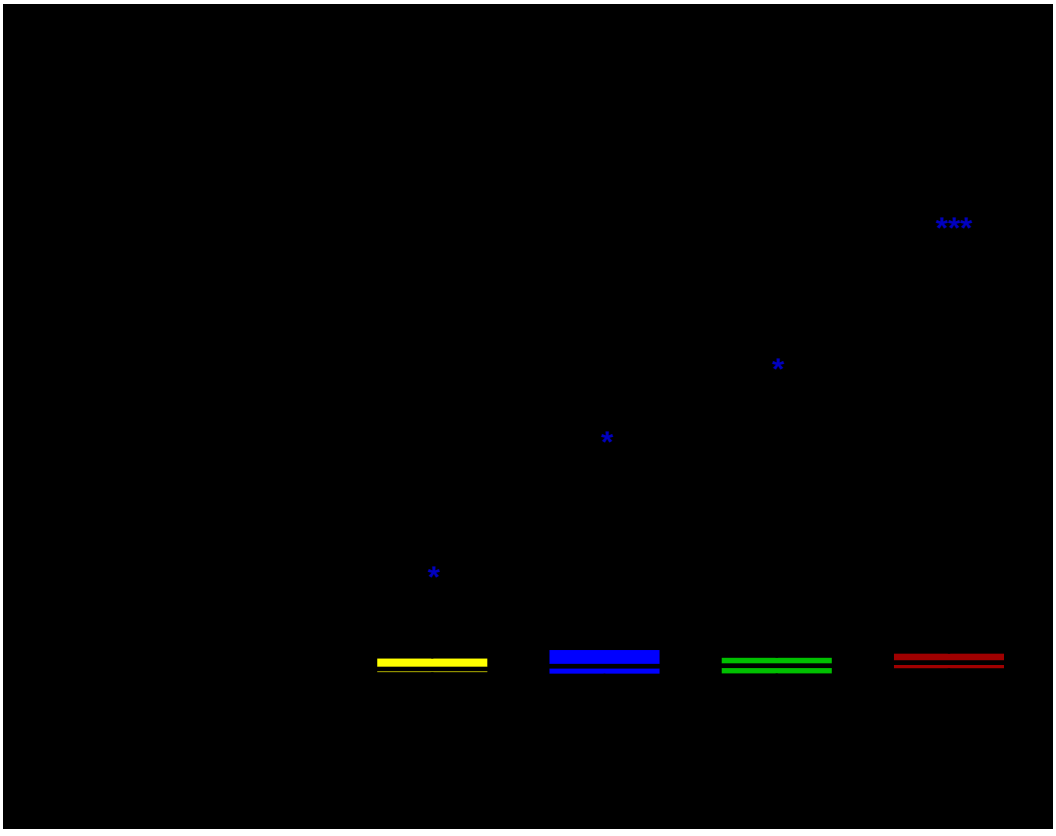


Figure 3.4: Bars are means of CPM; the error bar is standard error of means (SEM). The lines in the boxplots represent the median value. Significant difference are shown by * (P<0.05), ** (P<0.01) and *** (P<0.001). PTG is peptic tryptic gluten; PepG is ω-gliadin/C-hordein; peptide PepR is rye-derived peptide; PTH is peptic tryptic barley hordein.

The medians (SI) obtained from the all tested antigens were compared with the median (SI) of PTH (Figure 3.5). PTH served as positive control to demonstrate the specificity of the T cell lines that was used in the cultures in the beginning. The SI to PTH was between 2.1 to 12.43 (median=3.75). Positive responses to the ω-gliadin/C-hordein peptide and the rye–derived peptide were displayed in most of the barley hordein small intestinal T cell lines, with SI varying between 2.77 to 30.85 (median = 3.55) and 2.04 to 40.71 (median = 3.69), respectively. Cross-reactivity was demonstrated between barley hordein sensitive T cell lines with PTG (n=9), the S.I ranging from 2.25 to 59.6 (median = 7.4) Responses to the substrate PHA (data not shown) were high, indicating that the cells were proliferating in response to this mitogen. Kruskal Wallis test ($P<0.05$) with Dunn's multiple comparison post test determined that the proliferative responses of both peptides were not significantly different from PTH, showing that both peptides were as immunogenic as the positive control, PTH.

Figure 3.5: Comparison of the SI for each tested antigen

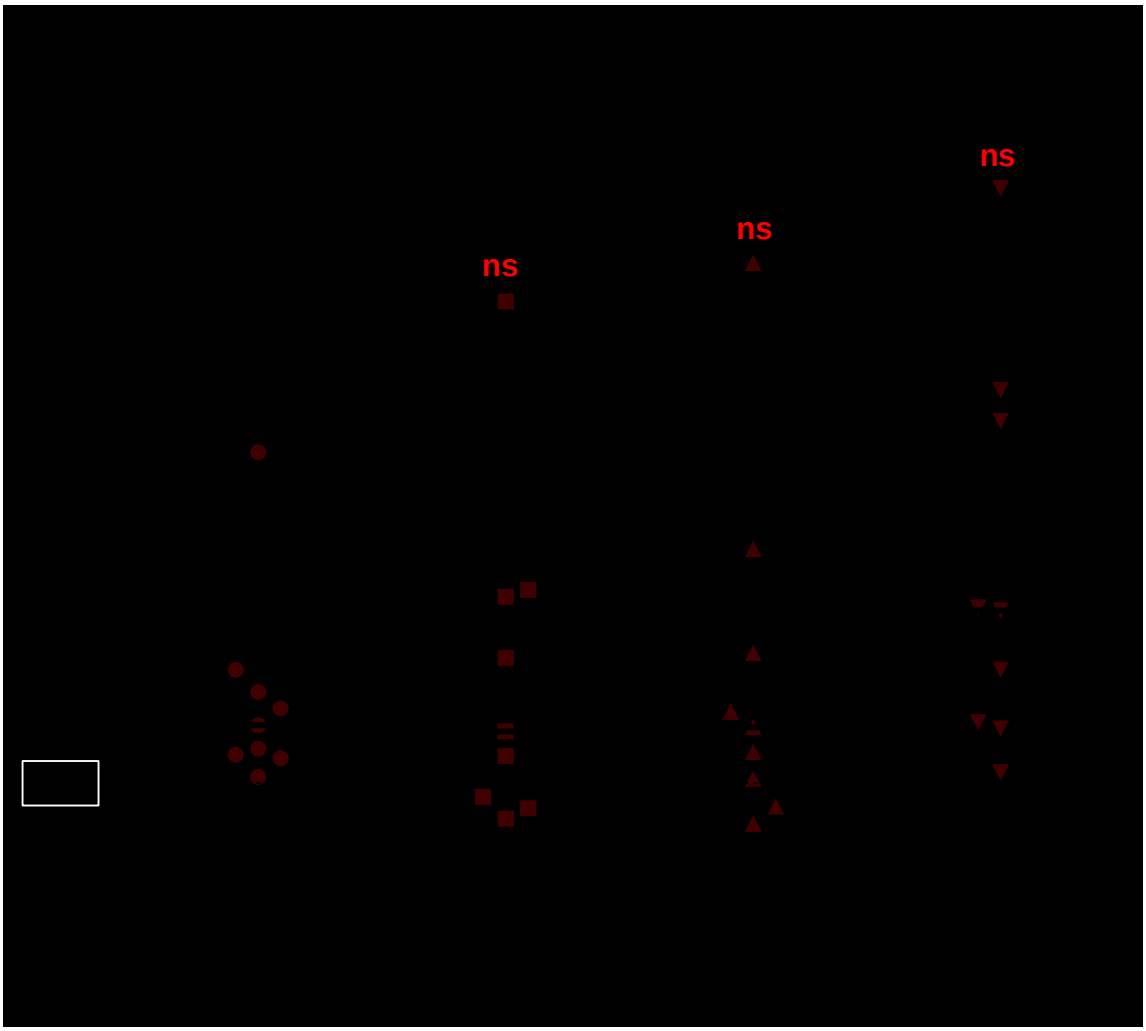


Figure 3.5 Each dot represents an SI for an individual assay. The lines represent the medians of the value for each tested antigen, **ns** indicates not significant. PTG is peptic tryptic gluten; PepG is ω-gliadin/C-hordein; peptide PepR is rye-derived peptide (QPFPQPQQPIPQ); PTH is peptic tryptic barley hordein.

The response of individual patient's barley hordein sensitive small intestinal T cell lines to all tested antigens is shown in Figure 3.6. An SI for WG5 is markedly higher than for all other T cell lines. The proliferative responses of WG5 to PepG and PepR are 5 times higher than the average. The majority of T cell lines positively responded to all tested antigens (SI between 2.04 to 59.6). T cell lines of WB4 and WB9 did not respond to either peptide, but only to PTH and PTG.

Figure 3.6: The SI for individual patient to all tested antigens (barley hordein sensitive small intestinal T cell lines)

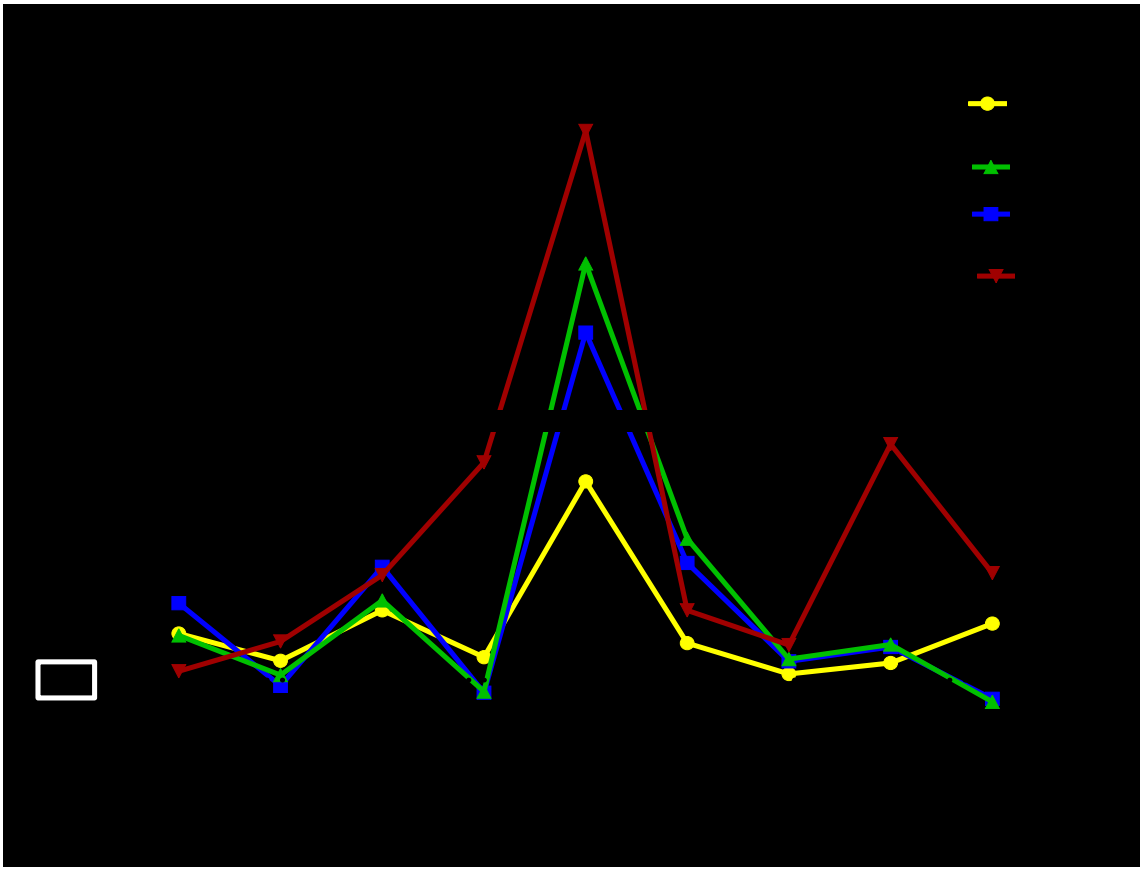


Figure 3.6. Each line corresponds to SI obtained per antigen tested with 9 T cell lines, labelled with WB1-9. A dotted line at SI of 2 distinguishes positive from negative results of proliferation assays. PTG, peptic tryptic gluten; PepG, ω-gliadin/C-hordein peptide; PepR, rye-derived peptide; PTH, peptic tryptic barley hordein

3.5.4 Generation of rye secalin small intestinal T cell lines

Only 2 small intestinal T cell lines sensitive to rye secalin were established, indicated by positive SI of 2.37 and 6.21 to PTS. These lines were obtained from co-culturing the biopsies of CD patients for 18h in the presence of PTS (5mg/ml) to sensitise the cells. As the lines were raised to PTS, that antigen was the positive control. As shown in Table 3.4, the results are expressed as arithmetic mean of CPM ± SEM, the other column shows the calculated SI.

Table 3.4: Proliferation assays of rye secalin (PTS) small intestinal T cell lines

Patient no.	R (s)	T cells +APC **No antigen**	T cells +APC **PTS**		T cells +APC **PepG**		T cells +APC **PepR**		T cells +APC **PTG**	
		Mean CPM ±SEM	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI	Mean CPM ±SEM	SI
WR1	3	721 ±135.5	1707 ±152.5	2.37	2403.33 ±181.7	3.33	2010.33 ±212.4	2.78	967.33 ±181.6	1.34
WR2	2	1534.13 ±125	9522.7 ±423.5	6.21	15697.7 ±586.7	10.2	2596 ±99.7	1.69	17159.3 ±1147.2	11.2

Table 3.4 Tested antigens are PTG, ω-gliadin/C-hordein (PepG), rye-derived peptide (PepR), and PTS. R(s) is number of restimulations, CPM is counts per minutes, SEM is standard error of mean and SI is stimulation index.

The response of rye secalin T cell lines to each tested antigen is also shown in Figure 3.7. Comparison of medians between the tested antigens and PTS was not calculated as only two lines were established. Positive responses to the ω-gliadin/C-hordein peptide and rye-derived peptide were displayed in WR1 small intestinal T cell line only, with SI of 3.33 and 2.78, respectively. Surprisingly, the stimulation index to PTG, of the same T cell line has demonstrated a negative response.

Figure 3.7: The SI for each tested antigen with rye secalin sensitive T cell lines

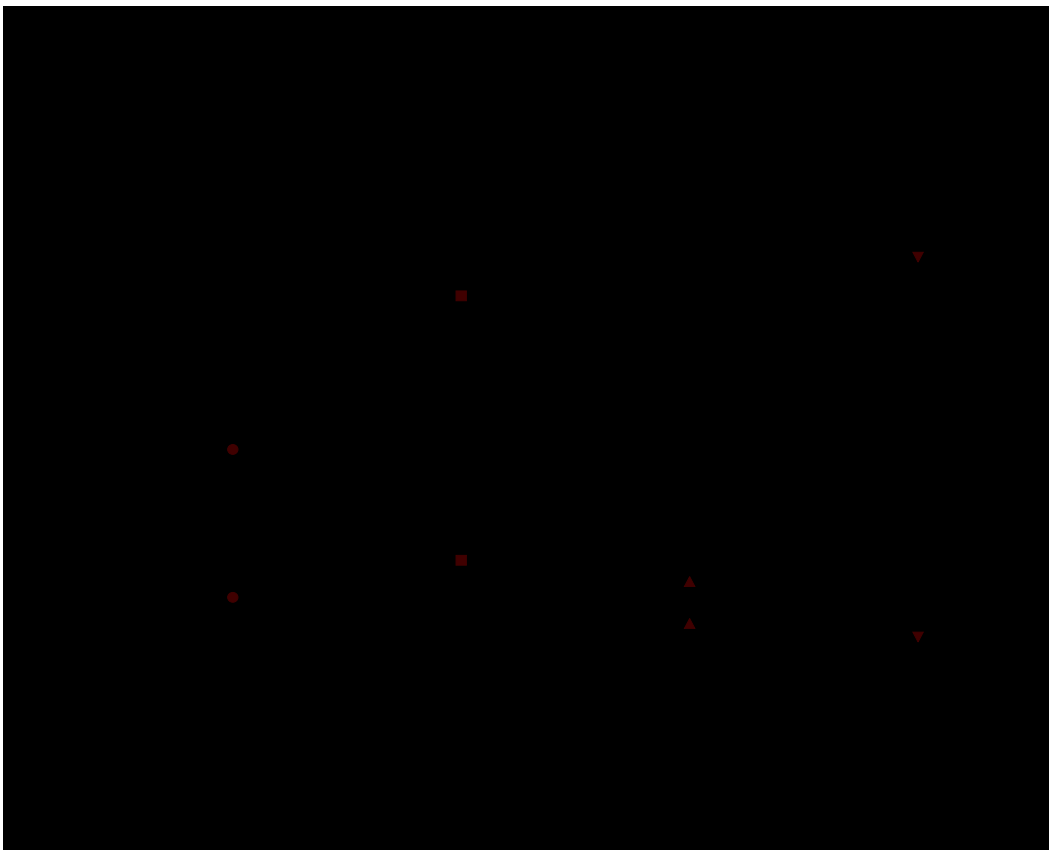


Figure 3.7 Each dot represents an SI for an individual assay. PTG is peptic tryptic gluten; PepG is ω-gliadin/C-hordein; peptide PepR is rye-derived peptide; PTS is peptic tryptic rye secalin.

3.6 Discussion

The primary purpose of this work was to investigate the coeliac toxicity of cereal prolamins peptides using gluten sensitive small intestinal T cell lines. Candidate peptides derived from cereal prolamins of wheat, rye and barley were obtained from the literature (Tye-Din 2010) and are thought to be immunogenic and exacerbate CD. In this piece of work, the establishment of *in vitro* assays on small intestinal T cell lines is presented together with the assessment of the selected peptides.

In some in vitro T cell studies, the investigation of coeliac toxicity of gluten fractions and peptides were undertaken with wheat gluten sensitive T cell lines, as wheat is consumed widely in the most populations. Kilmartin (2006) generated gluten specific small intestinal T cell lines by co-culturing them with gliadin, and then restimulated them with gliadin, glutenin, barley hordein and rye secalins fractions. The antigenic reactivity of T cell lines restimulated with barley hordein and rye secalin might have been assisted from the earlier sensitivity to CD toxic wheat gliadin.

In this study, we preferred to establish specific lines by directly co-culturing with PTG or PTH or PTS as stimulating antigens. PTG, as opposed to gliadin, was used as the stimulating antigen in the majority of individuals with CD. This was because we assumed that there are antigenic determinants present in HMW and LMW glutenins (Molberg 1998, Dewar 2006) that would be missed if gliadin alone was used. Initially, the filtered cells were not given added IL-2, so that the sensitisation of the cell lines was exclusively due to the presence of the antigen. The cells were also exposed to non-deamidated antigen assuming that the endogenous tTG within the small intestinal biopsies would be released and active in the tissues (Molberg 2001). In wheat gluten small

intestinal T cell lines, PTG was appointed as a positive control to compare other tested antigens. PTG was also an indicator of whether the established T cell lines were antigen-sensitive. We can confirm the generation of gluten sensitive T cell lines, not only because gluten was used to challenge the cells at initial stage, but also the SI has shown positivity.

Besides PTG, T cell lines were derived from CD small intestinal biopsies to cereal prolamins of barley (PTH) and rye (PTS). This is the first study, to our knowledge, utilising this approach, and has demonstrated the ability of these prolamins to activate T cell responses in small intestinal T cell lines obtained from duodenal biopsies from individuals with CD. It is interesting to demonstrate the potential of barley and rye to sensitise small intestinal immune responses at an initial stage.

The findings from this study further confirm that these related cereal peptides are equally CD toxic since they demonstrated similar effects to T cell lines generated with PTG. Cross-reactivity was between (i) the wheat gluten sensitive T cell lines with PTH (n=10) and PTS (n=9), (ii) the barley hordein sensitive T cell lines with PTG (n=9); (iii) the rye secalin sensitive T cell lines with PTG (n=2). The toxicity of these prolamins is also indicated by the fact that majority of the T cell lines described above demonstrated immunoreactivity to ω-gliadin/ C-hordein peptide and the rye secalin-derived peptide. The cross-reactivity shown by these lines to both peptides provides further evidence of the potential presence of these peptides in the Triticeae prolamins. This explains why barley and rye should continue to be excluded in a GFD. The SI results also revealed that responses to PTG are generally stronger than to PepG and PepR alone. This could be due to the fact that PTG is comprised of abundance of immunostimulatory gliadin and glutenin peptides.

The T cell responses to both peptides and PT prolamins may vary between individuals with CD (Figure 3.2 and 3.3). Gluten sensitive T cell lines of patient WG4 and WG10 were found to be significantly high, although the response was assessed only after one (WG4) or two (WG10) antigenic restimulation(s). In patient WG4, the stimulation index to PTG is ten times greater than the average SI with gluten sensitive T cells from CD small intestinal biopsies. Both ω-gliadin/C-hordein peptide and the rye-secalin derived peptide exhibited a high level of CD immunogenecity. The proliferative response of this line (WG4) to PTH and PTS were also very high, indicating T cell line from this CD individual was very sensitive to Triticeae prolamins. This individual had taken a GFD for >15 years. The T cell lines obtained from individual WG10, exhibited significant cross-reactivity to all the tested antigens although low numbers of cells were observed after the first restimulation. This individual had commenced GFD two years previously after being diagnosed with CD. It is possible that these two individuals have greater sensitivity to the tested antigen compared to other patients. Not all studied patients are sensitive to both peptides, but the majority are.

Heterogenous responses were also observed with the barley hordein sensitive T cell lines (Figure 6). In patient WB5, the proliferative responses to PTG, peptide ω-gliadin/C-hordein and peptide rye-secalin derived peptide were high. This is unsurprising since wheat gluten-sensitive T cell lines from the same patient (WG4) showed high levels of cross-reactivity to all the tested antigens.

The T cell lines sensitive to rye secalin were limited to two. Attempts to establish lines were made several times but it proved challenging. For example, the WR1 line had to be restimulated thrice before a sufficient number of T cells could be obtained to undertake proliferation assays. Both T

cell lines specific to the rye secalin exhibited cross-reactivity to ω-gliadin/C-barley hordein peptide, but only one of the lines reacted with the rye-secalin derived peptide. This could be explained by the fact that wheat has been used more widely in the food industry, such that it has become more popular in the diet compared to rye and barley. This suggests that patients may be less frequently exposed to rye and barley antigens.

Since the prolamins and peptides are high in proline and glutamine, the antigens were exposed to tTG for deamidation to enhance their CD immunogenicity. Several studies have demonstrated the preference and recognition of gliadin peptides following the exposure to tTG. Deamidation generates negative charges in prolamins and peptides, converting glutamine (G) to glutamic acid (E), thus facilitating their binding to HLA DQ2 and DQ8 molecules. This is thought to contribute to the observed small intestinal responses in CD. Our initial experiments were unsuccessful probably because the peptides were not deamidated (data not shown). Tye-Din and colleagues (2010) used deamidated prolamins, preferentially recognised by peripheral blood T cells, and the peptides were also pre-treated with tTG. Our results in some way agree with Tye Din (2010), showing the immunogenicity of the selected peptides. However, the immune response elicited by these peptides is very high in some patients, and average in the rest.

CHAPTER FOUR: PRODUCTION OF MONOCLONAL ANTIBODIES TO IMMUNOGENIC PEPTIDES OF ω-GLAIDIN/C-HORDEIN AND RYE SECALIN

4.1 Introduction

Single clones of hybridomas secrete a product, known as MAb that specifically binds to a single epitope or antigenic determinant. Ever since the development of hybridoma technology in 1975 by Kohler and Milstein, MAbs have been widely applied in diagnostic and therapeutic fields as well as targeted drug delivery. MAbs can be made on a large scale since the hybridomas possess the property of indefinite life allowing propagation and growth in tissue culture. Hybridomas, originating from the hybridisation of splenocytes and myeloma cell lines, can also be frozen for storage and recovered if a new batch of MAb is required. The steps of conventional MAb production are illustrated in Figure 4.1.

In CD studies, MAbs are employed in ELISA, as the specific binding characteristics allow for the detection of immune triggering peptides in foods. This is one of the most reliable tools to screen the safety of foods specialised for coeliac sufferers. However, MAb production is not easy and required a number of considerations.

The first consideration is the animal used as an in vivo donor of lymphocytes. A variety of animals have been used as host, but the majority of MAbs are derived from Balb c mice (Mitea 2008, Moron 2008, Spaniej-Dekking 2004, Ellis 1994 and Ellis 1989). Balb c mice have been widely used to generate myeloma cell lines, making this a more suitable strain for hybridoma formation.

Figure 4.1: Conventional production of MAb

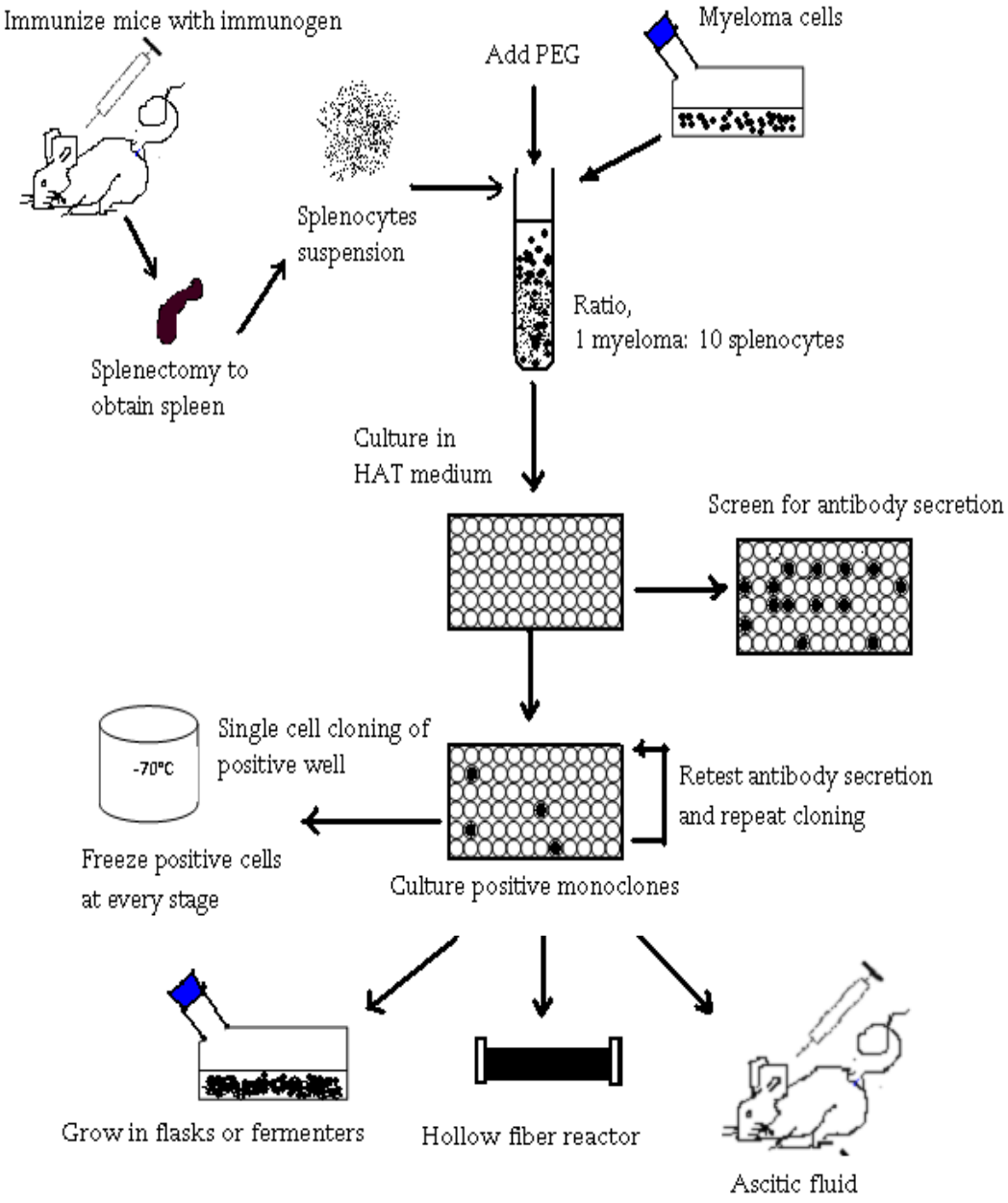


Figure 4.1 The illustrated diagram shows the steps in conventional hybridoma and MAb production (modified Eryl Liddell and Ian Weeks, 1995)

The second consideration is the immunisation that includes the immunogens, immunisation techniques and immunisation schedule. MAbs could be produced to particular CD epitopes or peptides, since these are recognised as foreign substances in the recipients. However, the peptide immunogens are

often too small to trigger sufficient immune response, thus they are normally conjugated with carrier protein.

Freund's adjuvant is the most commonly used reagent for the slow release of the immunogen from the point of injection. The presence of the *Mycobacterium tuberculosis* could augment the immune response of the recipient. For this reason, only the first dose of immunogen is emulsified in complete Freund's adjuvant (CFA). The subsequent immunisations should not incorporate these bacteria; instead incomplete Freund's adjuvant (IFA) is used.

Several techniques for producing antibodies to toxic peptides have been reported, particularly to wheat gluten. Moron et al (2008) raised MAbs to a purified plasmid constructed recombinant protein of 33-mer polypeptide α-gliadin (33-mer-THSP70 and 33-mer-X2-THSP70). The 33-mer peptide gliadin polypeptide was found to be CD toxic *in vitro* and *in vivo* (Shan 2002). The Balb c mice were subcutaneously immunised with a 25μg dose of immunogen, twice and intravenously for the third dose. In all immunisations emulsification in adjuvant was not used.

Spaniej-Dekking and colleagues sought to develop MAbs to α-gliadin (aa 59-71) peptide and γ-gliadin peptides (aa 142-153 and 147-159). Immunisations were carried out intraperitonealy with 150μg of peptide conjugated to tetanus toxoid (TTD) or bovine serum albumin (BSA), suspended in Freund adjuvant. Three following doses were given at the same concentration suspended in incomplete adjuvant (Spaniej -Dekking 2004). Similar attempts were followed years later which involved MAbs to glia-α9, glia-α20, glia-γ1, LMW glutenin-156 and HMW glutenin. All peptides were conjugated to TTD (Mitea 2008). Sanchez and co-workers (2007) raised MAbs that are able to recognize a number of antigenic epitopes of α-, γ- and -ω-secalin molecules. The latter

showed the highest number of antigenic epitopes recognized by the produced antibody. The immunisations involved two doses of 50-100 g of crude gliadin (Sigma) in CFA subcutaneously and a boosting dose intravenously without CFA. Skeritt et al (1985) produced a heat stable ω-gliadin MAb used to determine gluten in raw, cooked or processed foods (Skeritt and Hill 1990). The sandwich ELISA assay utilizing this MAb has been employed as the official method for gluten quantification by the Association of Analytical Communites (AOAC) (Thomson 2008).

The first MAb developed to non-wheat antigen was based on the ethanolic rye extract (Sorell 1998). The R5 MAb recognises a coeliac-toxic repetitive pentapeptide epitope (Osman 2001) that is present in all monomeric fractions of gliadin, barley hordein and rye secalin (Valdes 2003). The commercially avalaible detection assay kit based on this MAb has been adopted by Codex for quantification gluten content in foods (Codex Alimentarius 2008).

Professor Ciclitira's laboratory researchers successfully produced MAbs to gliadin peptides. The first MAb was raised against Frazer's Fraction III (Frazer 1959) by immunising Balb c mice which were restricted to GFD (Ellis 1989). A Bacillus Calmette-Guerin (BCG) vaccine was given to prime the mice as the following immunisations comprised of FIII conjugated to purified PPD. Later, MAbs to 54 aa (3-56) sequence of alpha-gliadin was developed (Ellis 1993) and demonstrated cross-reactivity to all toxic prolamins (Ellis 1994).

Several years later, a MAb was produced to a 19 aa peptide of A gliadin (31-49) (Ellis 1998). This peptide was reported to alter the mucosal architecture of CD patients in remission (Sturgess 1994), when infused *in vivo*. Moreover, the same group produced MAbs to immunodominant alpha-gliadin peptide (aa 57-75) that is known to be CD toxic (Anderson et al 2000, Fraser 2003) and also to

HMW glutenin (antibodies CDC3 and CDC7) found to be recognised in different wheat varieties (Dewar 2006).

The third consideration is fusion that is the key step of hybridisation. Splenocytes (spleen cells) are fused with mutant myeloma cells to ensure the antibody could be produced indefinitely. Myeloma cell lines are generated by culturing in the toxic analogue bromodeoxyuridine that selects thymidine kinase (TK) deficient cells, or in azaguanine that selects hypoxanthine guanine phosphoribosyl transferase (HGPRT) deficient cells. Important features of chosen myelomas for hybridisationa are i) they will not produce their own immunoglobulin and ii) they HGPRT or TK negative, that makes them incapable to survive in post-fusion hypoxanthine-aminopterin-thymidine (HAT) culture medium.

In fusion, it is possible to have the unfused and fused cells. Three types of fused cell populations could be: i) myeloma-myeloma, ii) splenocytes-splenocytes and iii) myeloma-splenocytes (hybrioma). Only the third population is able to survive in HAT medium, which has become the selection system to exclude the other irrelevant cell populations. The cells obtained from a successful fusion possess unique characteristics from the parents, first, being cancerous and therefore immortal (mutant parent myeloma) and second, having the potential to produce the desired antibody (lymphocytic splenocytes).

In the mechanism of HAT medium, the folic acid analogue (Aminopterin) is used to block the biosynthetic pathway for nucleic acid. The normal cells can continue to synthesize the nucleic acid using the salvage pathway. However, the mutant myeloma parent cell line which is lacking HGPRT, are not able to utilize the salvage pathway and cannot survive in the presence of Aminopterin

(A). The hybridomas (myeloma-splenocytes) are able to survive as the fusion supplies the HGPRT trait from the other parent, the normal splenocytes line. Hybridomas are further grown in HT after all control myeloma and splenocytes are dead.

The final stage is selection of hybridoma. This is done by screening the culture supernatant that might contain the desired antibody. If the hybridoma is found to produce antibody of interest, the cells will be cloned to produce MAbs. The monoclonal cell lines will be cultured for several weeks to increase the harvest of MAbs production. To some extent, the MAbs could be produced in larger scale for the use in immunoassays screening.

Chapter Three described in detail the immunogenicity of **ω-glaidin/C-hordein and rye-secalin peptides.** In this chapter, the development of MAb to those peptides is presented with a view to developing an improved assay for gluten quantification in foods designed for coeliac sufferers that will be further discussed in Chapter Five. The steps of MAbs production in this study were adapted from Professor's Ciclitira group using Balb c mice that were fed with gluten free food. The immunogens were conjugated to PPD by glutaraldehyde and emulsified in Freund's adjuvant. The employed immunisation schedule was based on Galfre and Milstein 1981, who proposed 3-5 weeks intervals, followed by a final intravenous injection without adjuvant, 3-4 days prior to fusion. The secondary subcutaneous injection was given four weeks after the first in the hope of obtaining the more useful class of IgG antibody, that is more stable and functional to be used immunoassays for gluten quantification.

4.2 Aim

We wished to develop MAbs to ω-glaidin/C-hordein and rye-secalin peptides**.**

4.3 Objectives

The objectives of this study are:

- To establish a methodology for successful immunisation of mice with PPD conjugated peptides
- To optimise the concentration of immunogen sufficient to induce an immune response
- To establish an immunisation schedule and optimise the interval time between the immunisations.
- To clone and characterise the MAbs to immunogenic peptides
- To study the possible cross-reactivity of the produced MAbs with other toxic proteins and peptides.

4.4 Methodology

The chemicals, equipments and the composition of all reagents are discussed in details in sections where required. The immunogens were derived from the same peptides described in Chapter Three.

4.4.1 Ethical consideration

These experiments were approved by the United Kingdom Home Office and carried out at King's College London (KCL) Biological Services Unit (BSU). The procedures were undertaken with the assistance from the supervisor under his Project Licence (70/19003) and Personal Animal Licence (70/6885). The animal work was classified as mild under the Home Office severity grading. The production of MAbs is covered under the Animals (Scientific Procedures) Act 1986.

4.4.2 Animals and GFD

Two breeding pairs of Balb c mice were obtained from a designated breeding establishment (Harlan) and placed together to produce offspring. The mice were fed with a purified gluten-free rodent diet (AIN-93 G, 829056) purchased from Special Diets Services, Dietex International Limited. The diet mainly consists of corn starch (39.75%), dextrinized starch (13.2%) and casein (20%). Previous study has demonstrated that antibody responses in animals that are not consuming gluten are significantly higher than in animals taking the standard rodent diet which contains gluten (Ellis 1998). Animals were cared for by the BSU staff, and inspected daily for any signs of distress and injury. Only one mouse died because of fighting. All of the mice were healthy and active throughout the series of immunisations.

4.4.3 Immunogens

The molecular size of peptide is one of the factors in choosing an immunogen. It was reported that low molecular weight antigens, that is less than 1000 Daltons (Da) are not good immunogens, generally. The peptides chosen for this study are synthetic peptides, which were the same peptides as used in the previous T cell study (Chapter Three). These peptides have demonstrated immunogenicity in the majority of wheat gluten and barley hordein sensitive T cell lines. In addition to both peptides, mice were also immunised with Peptide 3, an omega gliadin peptide in native form (1948 Da). The sequence of this peptide is similar to the sequence of wheat ω-gliadin/C-hordein peptide, except the 9th amino acid is glutamine (Q). The peptides are:

1) Wheat ω-gliadin/C-hordein peptide (1947.90 Da) labeled as **Peptide 1**-
   P Q Q P F P Q P **E** Q P F P W Q P

2) Rye-secalin derived peptide (1854.90 Da) or designated as **Peptide 2**-
   P Q Q P F P Q P Q Q P I P Q Q P

3) Wheat ω-gliadin/C-hordein peptide in native form (1948 Da) as **Peptide 3-**
   P Q Q P F P Q P **Q** Q P F P W Q P

It is interesting to investigate whether a single amino acid substitution, within a peptide, could potentially affect the efficiency of immunisation and subsequent production of MAbs.

4.4.4 Conjugation of peptide with PPD

It is necessary for the peptides to be conjugated to a larger carrier protein in order to generate sufficient immune response. The choice of carrier molecule and conjugating agent should be taken into consideration. Some examples of carrier proteins are ovalbumin, BSA, TTD and PPD. BSA tends to generate anti-BSA antibodies which might cross react in screening assays where BSA is used as a blocking reagent. Glutaraldehyde and carbidiimide are common conjugating agents used in MAb. Conjugation of carrier to peptide using carbidiimide (Goodfriend et al, 1964, Eryl Liddell and Ian Weeks, 1995) was reported to produce unstable conjugates; they could only last for a few days in solution. Glutaraldehyde (G5882, Sigma, Grade 1) was used in our study that cross-links covalently the amino groups of the peptide and carrier.

The tuberculin PPD (2T.U/0.1ml, 12-817-01, Statens Serum Institute), which is used in human Heaf testing was obtained from St. Thomas' Hospital Pharmacy. PPD contains glycerol and preservatives, and thus required purification prior to the conjugation to smaller peptides. The PPD was added to dialysis tubing (molecular weight cut-off 1000 Da, D7884, Sigma) and dialysed extensively with three changes of PBS at room temperature. Each dialysis against PBS was done for 45 minutes to remove glycerol. After dialysis, the volume of PPD would slightly increase since some of water molecules in PBS passed through the membrane. The final volume of PPD was measured (10.1 ml) and four times the volume of acetone (40.4ml) was added. The suspension was centrifuged at 3000rpm (1200 x g) for ten minutes after an hour at the temperature of -70°C.

The acetone supernatant was then decanted and the pellet was allowed to air-dry. PPD pellet was weighed; the molecular weight of PPD was assumed to be

10,000 Da. The molar ratio of PPD: peptide was calculated as 2:1. Each peptide and PPD were weighed out equivalent to this ratio and dissolved in PBS to give a concentration of 2mg/ml of PPD. Fresh, grade 1 gluteraldehyde (G5882, Sigma) was added to the solution at a concentration of 0.05% and stirred for 20 hours at room temperature. As PPD is light sensitive, the steps were protected from daylight, and the tubes containing the PPD-peptide conjugates were covered with aluminium foil during overnight stirring.

Following the overnight incubation, ammonium chloride (A4514, Sigma) was added to a final concentration of 0.1M and stirred for a further 30 minutes at room temperature. The conjugate was precipitated by adding four volumes of acetone at -70°C for an hour followed by centrifugation at 3000rpm (1200 x g) for 10 minutes. The supernatant was carefully removed, not to dislodge the conjugate pellet. The pellet was allowed to dry for around 30 minutes, weighed and dissolved in normal saline to the desired concentration. The PPD-peptide conjugate was divided into aliquots and stored at -20°C.

4.4.5 Preparation of immunogen- Freund's Adjuvant emulsion

A stable emulsion of immunogen in Freund's adjuvant was prepared freshly prior to immunising the mice. Gloves and eye protection were worn whilst handling Freund's adjuvant. Principally, adjuvants facilitate the slow release of the immunogen from the point of administration. CFA (64285, Lot 05410 MP Biomedicals) was used for the first injection. The second and subsequent injections required IFA (64286, Lot 9909H MP Biomedicals). Administration of both Freund's adjuvants containing the toxic peptide could induce the development of granuloma; hence increase the immune response of the animals. The emulsion was prepared with the total volume 200 µl per mouse. Equal volumes of Freund Adjuvant (CFA/IFA) and immunogen were drawn up with a 21-G needle (NN-2150R) and placed in separate 2ml

interchangeable all glass syringes (SYR2064). The syringes were joined by attaching to a 3-way tap, with the third nozzle closed-off. The syringes were held in each hand and the barrels were pressed against each other, in both directions, alternately. The pressure was applied on the barrel very quickly but carefully, repeated many times until a thick viscous milk-like emulsion was formed.

The stability of the emulsion was confirmed by applying a small drop to the surface of a 100-ml beaker of water. The drop of emulsion remained as a distinct droplet, and did not spread across the water surface. The stable emulsion (200 µl) was then transferred to 1ml Luer plastic syringe (300013, BD Plastipak) and capped with a shorter 21-G needle (C0114). A syringe was prepared for each individual mouse.

4.4.6 Immunisation procedures

The first generation of mice offspring were introduced to a GFD early in the suckling stage, from mothers that were maintained on GFD. The offspring continued the same diet at weaning stage (3 weeks of age). Prior to immunisations with PPD conjugate, the mice were primed with 100µl of BCG vaccine (0.2-0.8 million bacteria made in Diluted Sauton SSI, Statens Serum Institut), subcutaneously, at 6 weeks of age. This was performed by using a TerumoU-100 insulin syringe with needle (BS-01H2516). The first subcutaneous immunisation was performed two weeks after BCG vaccination. The immunogen was the synthetic peptide-PPD conjugate emulsified in Freund's adjuvant.

In previous work, antibody responses have been obtained after two immunisations. In this study, where titres were not adequately high, a further dose of peptide-PPD conjugate in IFA was given at 5 weeks later. A venous

tail bleed was undertaken 7-10 days after the last injection. Animals that exhibited the highest antibody titre were selected and given an intravenous booster injection (I.V) of 50μg peptide-PPD conjugate (without adjuvant) dissolved in 100μl normal saline. Mice were sacrificed 3-5 days later to harvest splenocytes, the optimal time when the relevant lymphocytes are at their peak of proliferation rather than antibody secretion. Splenocytes were used for fusion to create hybridomas, explained in detail in Section 4.4.9

4.4.7 Mice sampling

The first bred of Balb c mice successfully produced 15 pups from two litters. Only 11 mice were immunised with immunogens. The second batch of breeding delivered 12 pups with equal number of female to male offprings, all were immunised. The female and male resultant mice were separated into four cages, and each mouse was labelled by making an ear notch (left, right, both or no notch). The same way of sampling was applied when the second batch of litter were delivered. Since three or four mice were required for each of the peptide, the remaining mice were sacrificed where the splenocytes were harvested and used as feeder cells (see Section 4.4.11). Unimmunised mice were also culled as a prelude to undertaking fusion experiment.

The distributions of mice and immunisations procedures are simplified in Table 4.1 and Table 4.2

Table 4.1: The immunisations programme in the first breeding (first attempt)

Mouse	Sex	Peptide	Preliminary dose (route)	Second dose (route)	Third dose (route)	Final booster injection (route)
M1	Male	3	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No
M2	Female	1	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	50 µg in 100 µl (i.v)
M3	Female	1	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No
M4	Female	2	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No
M5	Female	2	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No
M6	Male	1	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	No
M7	Male	1	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	No
M8	Male	2	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	No
M9	Male	2	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	No
M10	Male	3	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	No
M11	Female	3	300 µg in 200 µl (s.c)	300 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	50 µg in 100 µl (i.v)

Table 4.2: The immunisations programme in the second breeding (second sttempt)

Mouse	Sex	Peptide	Preliminary dose (route)	Second dose (route)	Third dose (route)	Final booster injection (route)
M12	Female	1	1000 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No	No
M13	Female	2	1000 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No	No
M14	Female	3	1000 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No	No
M15	Male	1	1000 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No	No
M16	Male	2	1000 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No	No
M17	Male	3	1000 µg in 200 µl (s.c)	1000 µg in 200 µl (s.c)	No	No
M18	Male	1	600 µg in 200 µl (s.c)	600 µg in 200 µl (s.c)	No	50 µg in 100 µl (i.v)
M19	Male	2	600 µg in 200 µl (s.c)	600 µg in 200 µl (s.c)	No	50 µg in 100 µl (i.v)
M20	Male	3	600 µg in 200 µl (s.c)	600 µg in 200 µl (s.c)	No	No
M21	Female	1	150 µg in 200 µl (s.c)	150 µg in 200 µl (s.c)	No	No
M22	Female	2	150 µg in 200 µl (s.c)	150 µg in 200 µl (s.c)	No	50 µg in 100 µl (i.v)
M23	Female	3	150 µg in 200 µl (s.c)	150 µg in 200 µl (s.c)	No	50 µg in 100 µl (i.v)

4.4.8 Tail bleed and booster immunisation.

The blood was collected from the immunised mice to assess the immune response, either the mice were adequately immunised or required further dose of immunisation. Each mouse was allowed to enter an appropriate size of restraint tube, which only left the tail outside for venous tail bleed and booster immunisation. The restraint tube was washed and wiped thoroughly to prevent cross-infection and possible stress induced by pheromone released by earlier mice. The tail was milked gently to encourage blood flow. A small nick was made at the lateral tail vein, approximately one-third along the length of the tail from the tail tip.

The operator used a different gamma sterilized stitch cutter (0420, Swann Morton) for each tail. The blood was directly transferred to a micro-centrifuge tube and labeled. The blood sample was collected in the range of 10-25 µl. If the volume of blood was inadequate, any blood clots were wiped with a clean tissue, and another nick was made towards the base of the tail. Blood flow was stopped by applying finger pressure on soft tissue placed at the blood sampling site for approximately 30 seconds before mice were returned to their cages.

The blood samples were immediately spun down in a mini-centrifuge at maximum speed to separate the serum. The serum was aspirated using 10-100 µl micropipette, and the volume was recorded. The serum was diluted with 100 µl of PBS and kept frozen in -20C. The mice were checked after 30 minutes and the next day for their general health and appearance. The sera were tested for antibody production using a simple indirect sandwich ELISA (see Section 4.4.9).

Mice with positive sera shown in ELISA assays were inoculated with a final booster immunisation using a restrained tube was used to ease the procedure.

Prior to the intravenous injection, the tail was warmed with a bulb to encourage vasodilatation of blood vessels. The tail vein was cannulated slowly and carefully using 25 G (NN-2516R) short needle. Booster injection was undertaken to maximize antibody production, act as a further secondary immunisation of peptide conjugate.

4.4.9 Assessment of antibody responses using indirect sandwich ELISA

Indirect sandwich ELISA was mainly used in our study to test the sera collected from immunised mice, screen the hybridoma supernatant post-fusion and analyse the hybridoma supernatant of new clones. The method was a standard protocol undertaken by a previous researcher in our group, adapted from Kiefer (Kiefer et al. 1992). The volumes of coating antigen, primary and secondary antibody used were 50µl, washings were 250µl. Polystyrene 96-well NUNC plates were coated with solubilised antigen for 18 hours at 4°C. Coating antigens were: PTG in carbonate buffer at 25 and 50µg/ml, Kolibri gliadin in 60% ethanol at 50µg/ml or BSA in PBS/ tween 20 at 50mg/ml, the latter used as a negative control coating antigen. Since the blocking reagent contained BSA, this was to ensure that antibodies were not binding to the blocking agent.

Following the overnight coating, the contents of wells was discarded and the unbound antigen was washed thrice with PBS/ 0.05 % Tween 20. Blocking solution was then added, incubated at 37°C for 1 hour and washed once after the incubation. Test sera and positive control MAb supernatants previously made by our group such as anti-gliadin 31-49, MAb PN3 (Ellis 1998) and anti-gliadin 56-75, MAb CDC5 were diluted in serial doubling dilutions over the range of 1:100 to 1:102,400. The MAb supernatants were used over the range of 1:2 to 1:4096. The last column of PBS served as background in determining the cut-off point.

For the initial screening of hybridoma supernatants and new clones, the saved culture media was not diluted.

The plates were incubated for 45 minutes at 37°C. The unbound materials were washed away by rinsing the wells thrice with 250µl of PBS/0.05% Tween 20. The plates were drained by patting hard on paper towels. Rabbit anti-mouse IgG (A4312, Sigma) or goat anti-mouse IgM (A9688, Sigma), conjugated to alkaline phosphatase was used as secondary antibody prepared by diluting 1:2000 in PBS/0.05% Tween 20. The plates were again incubated for 45 minutes at 37°C, washed three times and drained.

The final step was the addition of alkaline phosphatase liquid substrate pNpp (p7998, Sigma). The plates were placed in the dark at room temperature, and left for the colour to develop. After 30-45 minutes, O.D was read using Titretek Multiscan plate reader at 405 nm. The lowest concentration of serum was determined by choosing the O.D greater than 1.5 times the average of six blank wells (background).

4.4.10 Myeloma cell retrieval and preparation

P3X63Ag8.653 (Health Protection Agency Culture Collections) myeloma cells were retrieved from liquid nitrogen storage approximately three weeks before fusion to allow the cells to be in the log phase of growth. Prior to the retrieval 20 ml of RPMI medium was placed on ice for about an hour. Gloves and goggles were worn whilst handling samples from liquid nitrogen. Cryotubes containing cells were retrieved from liquid nitrogen storage, quickly warmed under running hot tap water. Once the ice pellet appeared to dislodge from the walls, the tubes were run under tap water until the content became partially liquid. The contents

were transferred into 15 ml Falcon tube, 1ml of cold (4°C) RPMI medium was added drop-wise, followed by the addition of 9ml RPMI. DMSO (D2650, Sigma) used for freezing the cells is detrimental at temperatures above -70°C, thus the retrieval was undertaken swiftly at the temperature as low as possible. The tube was centrifuged at 300 x g (1500rpm) for 7 minutes and the supernatant was discarded. The cell pellet was washed once again with 10 ml of RPMI medium and added with 2 ml of fresh culture medium, transferred to 1 well of 24-well plate and left undisturbed for several days. This step is to ensure the live cells would have adequate cell-cell contact to multiply. When the cell density had increased, the cells suspension was expanded to few more wells and added with fresh medium. The cells were transferred to 50 ml culture flask.

4.4.11 Cell culture and maintenance

Myeloma cells were cultured in horizontal flasks for approximately three weeks before being used in fusion. Often, many cells adhered to the walls of culture flasks and required scrapping prior to refreshing the culture medium. The myeloma culture medium was given an elimination dose of plasmocin (25μg/ml) as the cells lines were established from frozen myelomas. The maintenance dose (12.5μg/ml) was added to hybridoma cell cultures given that the cells were nascent and maintained at longer period. Bacterial contamination was avoided by adding antibiotic besides sterilizing apparatus with gamma irradiation source (500Gy). Penicilin/streptomycin (100μg/ml) and gentamycin (200μg/ml) were added to the culture medium. Penicilin/streptomycin is affective against Gram-positive and Gram-negative bacteria, respectively. Gentamycin is effective against a broader spectrum of both Gram-positive and Gram-negative bacteria and can overcome some strains that might have developed resistance to penicilin/streptomycin.

Fungal contamination could be prevented by the use of amphotericin B (2.5µg/ml) in the culture medium. However, the cell culture should be discarded immediately if it occurs, since the contamination could be easily spread to other flasks due to airborne spores. All the cultured cells were kept in an incubator with an atmosphere of 5% CO2. When the colour of culture medium changed from red to yellow, that showed the fall of pH and the depletion of nutrients in the medium, cells were then normally fed and the old medium was replaced with new fresh medium.

4.4.12 Media preparation

Prior to undertaking the splenectomy, several types of media were prepared. RPMI 1640 was mainly used as in the formulation of tissue culture media, as well as washing and re-suspending medium. HAT (H0262, Sigma) medium was used for cell selection after fusion of myeloma cells with splenocytes. After 5-7 days, when all cells were observed dead, except the surviving myeloma- splenocyte hybrids, HAT was replaced with HT (H0137, Sigma) medium. HAT and HT lyophilized powder were initially dissolved in 10ml of double processed tissue culture water (W3500, Sigma), before preparing the required media. The formula for each type of medium can be referred in the Appendix I section.

4.4.13 Splenocyte harvesting

The technique for splenocyte harvesting and cell fusion is extremely laborious. For each attempt, only one mouse was sacrificed to harvest splenocytes. The culling and dissection procedures were undertaken at the BSU, assisted by the staff. The remaining steps after obtaining the spleen were performed in laminar flow safety cabinet. The mouse was killed by cervical dislocation and the fur was

drenched in absolute alcohol. A midline incision was made to the skin and abdominal wall, using fresh sterile instruments for each stage, and the layers pinned back. The sternum was dissected to open the thoracic cavity. The murine heart was transected, and the pooled blood was aspirated and transferred into a centrifuge tube, rapidly before further dissection to avoid blood clotting. The serum was obtained after centrifugation using the mini-centrifuge at maximum speed. The serum was then frozen for use as a positive control in future screening assays. The peritoneum was cut to expose the abdominal viscera.

The spleen was blunt dissected and placed immediately in double strength antibiotic medium for 30 minutes. In a laminar flow hood, the spleen was washed in single strength antibiotic medium. Two ml of single strength antibiotic medium was added to the spleen and both were poured carefully into a Potter-Elvehjem tissue homogeniser. The 3 strokes of the pestle were used to pulverize the spleen and release the cells. The cell suspension was transferred to a 15 ml Falcon tube using a squeezing pipette and any large debris was left in the mortar. The tube was centrifuged at 300 x g for 10 minutes. The supernatant was discarded and the cell pellet was re-suspended in 5ml of red cell lysis buffer-Hybrimax (R7757, Sigma) to lyse the erythrocytes followed, by under-layering with 2ml of FCS. This cell suspension was again centrifuged 300 x g for 10 minutes and the pellet was re-suspended in 5ml of serum-free medium. The supernatant containing the contents of the lysed erythrocytes was removed. The tube containing the harvested splenocytes was placed on ice before fusion was undertaken.

4.4.14 Fusion of splenocytes with myeloma cells

Fusion was performed subsequently after splenocyte harvesting, in a laminar flow safety cabinet. A mouse spleen is estimated to have 1 x 10^8 splenocytes. Therefore, myeloma cells (P3X63Ag8.653), required are between 2 to 10 x 10^7 per spleen. This estimation is based on the ratio of 2-10 splenocytes to one myeloma cell to get an optimal fusion. A 20µl aliquot from 15ml myeloma cell suspension was mixed with 20µl of 0.45% trypan blue dye and applied onto the chamber of an improved Neubauer haemocytometer.

Cells were observed under microscope, only the viable cells that excluded the dye and appeared round and shiny were counted. Once a satisfactory count and harvest had been performed, myeloma cells were washed thrice in RPMI medium to eliminate all traces of FCS, which might interfere with fusion, and re-suspended in 5ml of RPMI medium. A one ml aliquot of the myeloma cells was saved in a separate well to use as an unfused myeloma cell control well and, another control well was included, containing the unfused splenocytes only.

The myeloma cell suspension was added to the spleen cells and washed again three times in RPMI medium to remove the remaining FCS. The cell mixture was pelletted by centrifugation at 300 x g (1500rpm) for 10 minutes and the supernatant was thoroughly aspirated using a pipette. The dry cell pellet was re-suspended in 1 ml of PEG (P7181, Sigma, hybrimax grade) solution, pre-warmed at 37°C. PEG disrupts the cell membrane of the cells by reducing the surface tension permitting the fusion of cell membranes. The mixture was vortexed for 2 seconds and held in warm hands for 90 seconds with continuous gentle agitation.

Next, one millilitre of complete medium was added drop-wise, with constant gentle shaking. A further 9ml of complete medium was added slowly to dilute

PEG and stop the fusion process. Because of its toxicity to cells that could interfere with cell proliferation, any traces of PEG after fusion were eliminated by washing. The mixture was centrifuged at 150g (800rpm) for 5 minutes and the supernatant was discarded. The speed and duration of centrifugation at this stage were reduced owing to the fragility of the cell after fusion. The cell pellet was re-suspended in 10 ml of pre-warmed HAT (H0262, Sigma) medium and aliquoted 1ml/well in 24-well culture plates. To both control wells of unfused myeloma and spleen cells 1ml of HAT medium was added. These acted as controls for the effectiveness of HAT, in the presence of which both type of cells should die. The plates were sealed in polythene bags to aide prevention of contamination, whilst allowing slow diffusion of gas, and placed in the incubator with an atmosphere of 5% CO_2.

4.4.15 Culture of hybrids after fusion

After fusion, the cells were allowed to grow undisturbed for 7 days. Then plates were examined every 1-3 days to check the emergence of nascent hybrids, the changes in medium colour, and the presence of any contamination. Comparisons were made between the unfused myeloma controls well, unfused splenocytes control well and all wells containing the potential hybrids. Unfused splenocytes and myeloma are mortal and normally die after one week. If control cells were still alive, exposure to HAT was continued by adding approximately 1ml of HAT medium to all wells. Usually 7 days after initial fusion, all the cells in control wells were dead and the hybrids could be observed as small clumps. The hybrids were fed by adding 1ml HT medium, carefully so as not to disaggregate the colonies. The growing hybridomas were monitored over several weeks. Some wells would have non-hybridoma fibroblast-like cells that grew very rapidly and exhausted the nutrients in the medium. The yellowing of the medium was not

the only indicator showing the presence of hybrids. Therefore, cells were checked microscopically and size of hybridoma clonies was noted.

4.4.16 Screening of hybridomas

Although a fusion might be successful not all cells may produce the desired antibody. Each well comprised a pool of different hybrids that could produce non relevant antibodies. The objective of screening is to identify interesting hybrids and reject the negative wells. One hundred fifty microlitres of supernatant from each well was pippeted into microcentrifuge tubes and added with 30µl of N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) (H3375, Sigma), to maintain the supernatants at pH 7.4 for the ELISA. The ELISA assays were performed using PTG or PTH as coating antigens. The class of antibody from each screened supernatant could also be assessed, by the addition of anti-IgG/ IgM secondary antibody as the detecting agent. Supernatants with positive O.D were chosen for single cell-cloning, particularly those with the highest ELISA reading but appeared as the smallest colonies. The assessment was relatively subjective. The previous researcher had chosen 0.8 O.D as positive, compared to a background of 0.2. In this study, the background reading was very low (0.071) hence, an O.D of 0.6 and above was considered positive.

Hybridoma cells grow very rapidly with an exponential doubling time of 12-24 hours. Therefore, subsequent supernatant collections and screenings were performed at 3-4 day intervals, providing sufficient period for the antibody to be secreted into the culture medium to measure.

4.4.17 Cloning of positive hybridomas

During cloning, some of the positive cells were frozen down for future use, should the chosen samples yield unsatisfactory results. A previous researcher employed a very laborious single cell technique for cloning. In this study, cloning was undertaken by diluting the cell suspension to an average of 0.5 cells per well. The content of the chosen well was resuspended and the cells were counted in trypan blue dye, using Neubauer haematocytometer. A required volume of supernatant that provided 200 cells/ suspension was removed from the wells and pipetted into 50ml falcon tube. The small volume of suspended cells was added with 50ml of culture medium. The 200 cells/ 100ml medium were plated out into round-bottomed micro 250µl wells on a 96 well culture plate, with feeder cells prepared to 10^5 cells per ml: 200ul/well. The clones were left undisturbed for 10-14 days.

4.4.18 Screening of clones

Following cloning step after two weeks, culture wells were inspected under microscope for the presence of growing clones. Wells that contained only one emerging colony were noted. Fifty microlitre of supernatant from each well was removed and screened for antibody secretion by ELISA as described in Section 4.4.9.

4.4.19 Growth and expansion of clones

If antibody secreting clones were successfully generated, the clones were transferred to progressively larger vessels and supernatant will be saved. Some flasks of cells are fed regularly to ensure high cell viability, and cells will be harvested at regular intervals and frozen down. The student has previously grown

anti-gliadin PN3 MAb (Ellis 1998) successfully generated by the same group where there cultures were grown with a gradual decrease of FCS. This was to obtain high purity of MAbs without the presence of immunoglobulin from FCS. Supernatants were irradiated and stored at -20C. If required, the hybridoma supernatant can be precipitated to be used in immunoassays.

4.4.20 Precipitation of immunoglobulin

These experiments were undertaken to obtain concentrated and purified antibody. The medium containing cells was spun at 170 x g for 7 minutes at 24°C. The supernatant which contained antibody was saved after separating it from the cell pellet. The saved supernatant was transferred to a new tube. The volume was recorded. Next, equal volume of saturated ammonium sulphate (NH_4 $(SO_4)_2$) was added drop-wise. The colour of the solution became paler. The initial colour was slightly orange and then turned into yellowish. This indicated that the slightly acidic medium had become more acidic following the addition NH_4 $(SO_4)_2$ which is an acidic salt. This caused a fall in the pH. As more NH_4 $(SO_4)_2$ was added, the solution became cloudier indicating that the precipitation of protein was increasing. Finally, the solution was kept at 4°C for further precipitation overnight.

The next day, the solution was spun down at 800 x g for 30 minutes. The supernatant was discarded and the pellet was saved. PBS was added at 1/10 of the original volume of the antibody supernatant to resuspend the pellet. The solution was transferred to dialysis tubing, size 25mm x 16mm (Sigma D9777) for dialysis against PBS. The tube can retain most proteins of molecular weight 12000 Da or greater. The dialysis was carried out for 45 minutes on a magnetic stirrer, 3 times. Each time, the buffer was changed. The solution in the membrane was

finally transferred to a new 15ml Falcon tube, irradiated and kept at -20°C for future analysis.

4.4.21 Storage of cells

Cells were frozen down for long term storage once they were growing rapidly, in case they are required for future use. For example, the positive hybridoma cells, clones and myeloma cells. Cells were harvested, washed once in RPMI and resuspended in 1ml of culture medium. Cryogenic tubes, a freezing chamber, squeezing pipettes and freezing medium were placed on ice prior to the freezing process. The freezing mixture which comprised of culture medium with 20% DMSO was added drop-wise to the cell suspension to a ratio of 1:1. The final concentration of DMSO in the mixture was 10%. The cell suspensions were aliquoted in cryotubes (usually 1ml/ tube) and placed in a freezing chamber containing IPA. This was placed in the -80°C overnight and transferred to liquid nitrogen the following day for long term storage.

4.5 Results

This section describes the results of experiments in relation to the first and second attempt at monoclonal work. The first attempt of monoclonal work involved first breeding of mice (11 were immunised, 2 were unimmunised, 2 died because of fighting)

The second attempt was carried out with the second batch of resultant mice (all were immunised).

4.5.1 Immunisations

4.5.1.1 First attempt

The mice sera were tested by indirect ELISA with 25μg/ml and 50μg/ml PT gluten as the coating antigen. The details of the immunisation programme were previously explained in Section 4.4.7. The concentration for each dose was explained in Table 4.1. The sera assessment after the second dose is shown in Table 4.3. The ELISA detection dilutions after the third dose are shown is Figure 4.2, 4.3, 4.4, and 4.5. All the assessments were undertaken with anti-IgG secondary antibody.

Table 4.3: ELISA assessment (IgG), the titre of sera of mice after immunisation with peptides conjugated to PPD, compared to unimmunised mice

Supernatant	Plate with 25μg /ml PTG	Plate with 50μg/ml PTG
Positive control-	1/25600	1/25600
Negative control- Unimmunized mouse	nil	Not tested
M1-Peptide 3	nil	1/400
M2-Peptide 1	1/400	1/800
M3-Peptide 1	1/400	1/1600
M4-Peptide 2	nil	1/800
M5-Peptide 2	1/400	1/1600
M6-Peptide 1	nil	nil
M7-Peptide 1	1/400	1/400
M8-Peptide 2	1/400	1/400
M9-Peptide 2	1/800	1/800
M10-Peptide 3	1/400	1/400
M11-Peptide 3	1/800	1/400

Peptide 1= Omega gliadin/C-hordein peptide
Peptide 2= Rye secalin derived peptide
Peptide 3= Omega gliadin/ C-hordein peptide with Q substitution at 9th position
Positive control=PN3 MAb

A titre of 1/400, for example, means the highest dilution factor that allowed for readable absorbance reading. Second immunizations appeared to be insufficient to elicit immune response. Hence, the third dose with higher concentration of immunogen was inoculated (Table 4.1). Two mice were shown to be sufficiently immunised:

1) M2- titre of 1/ 25600
2) M11- titre of 1/12800

Figure 4.2: **IgG titre of PN3 MAb (Positive Control) and serum from unimmunised mouse (Negative Control), on a plate coated with 25 µg/ml PTG**

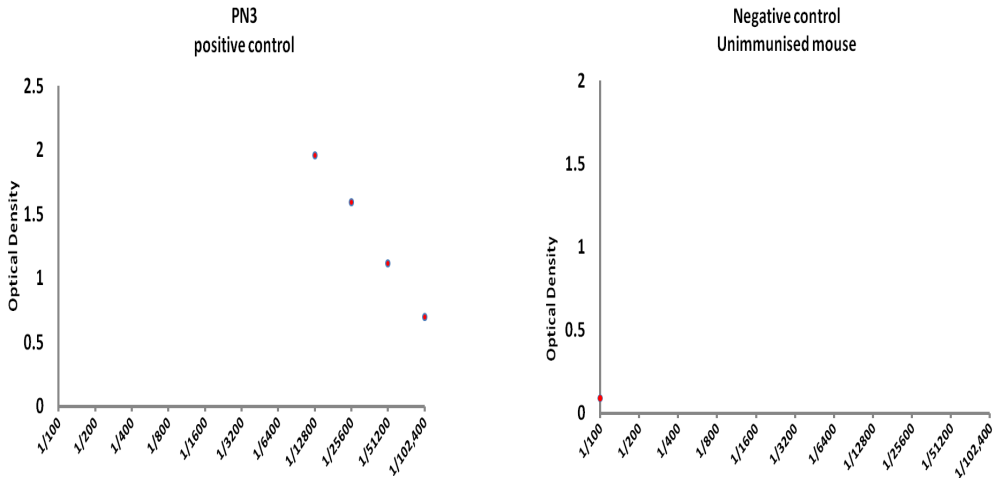
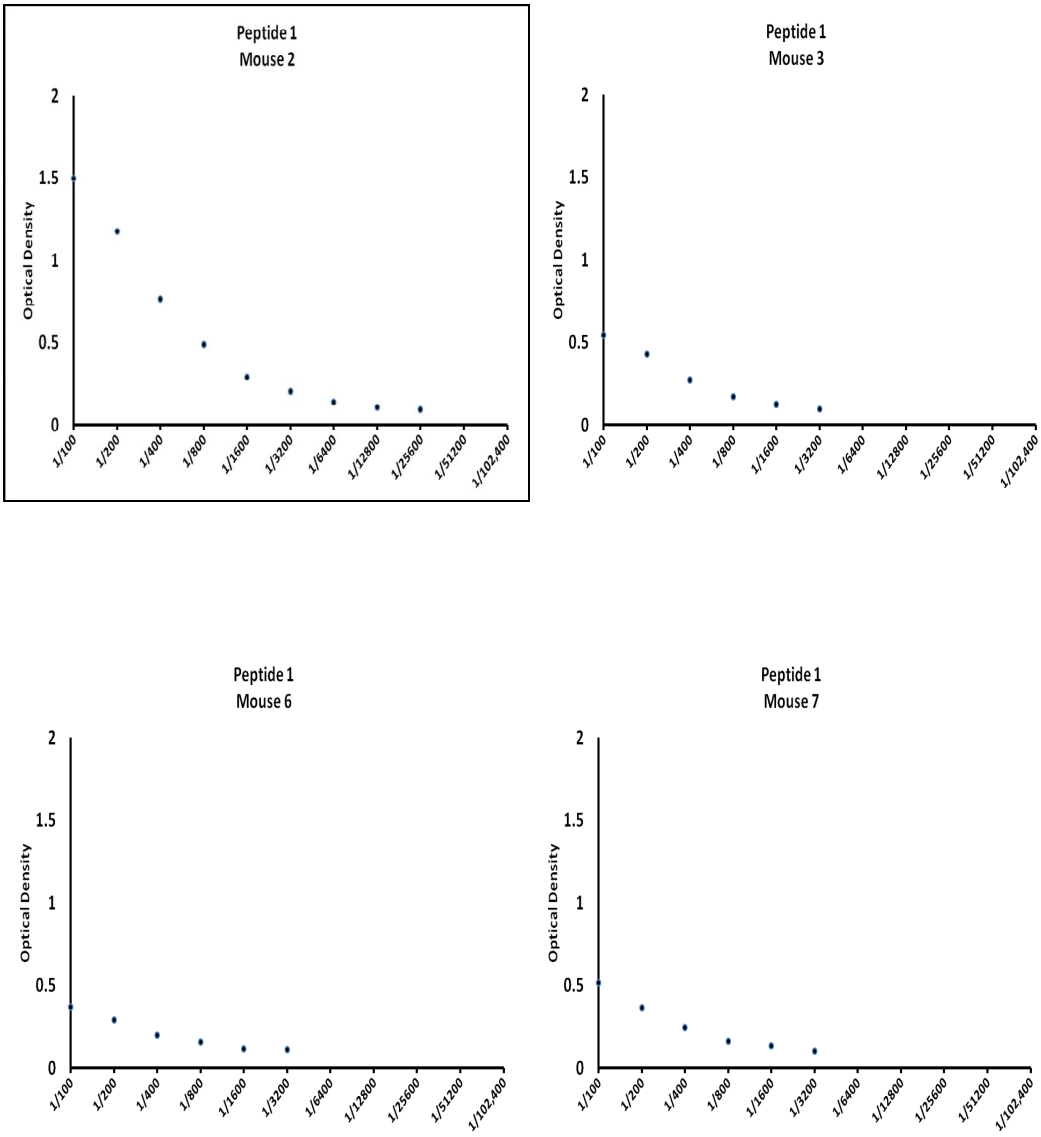


Figure 4.3: IgG titre of Peptide 1, immunised mice sera on a plate coated with 25μg/ml PTG

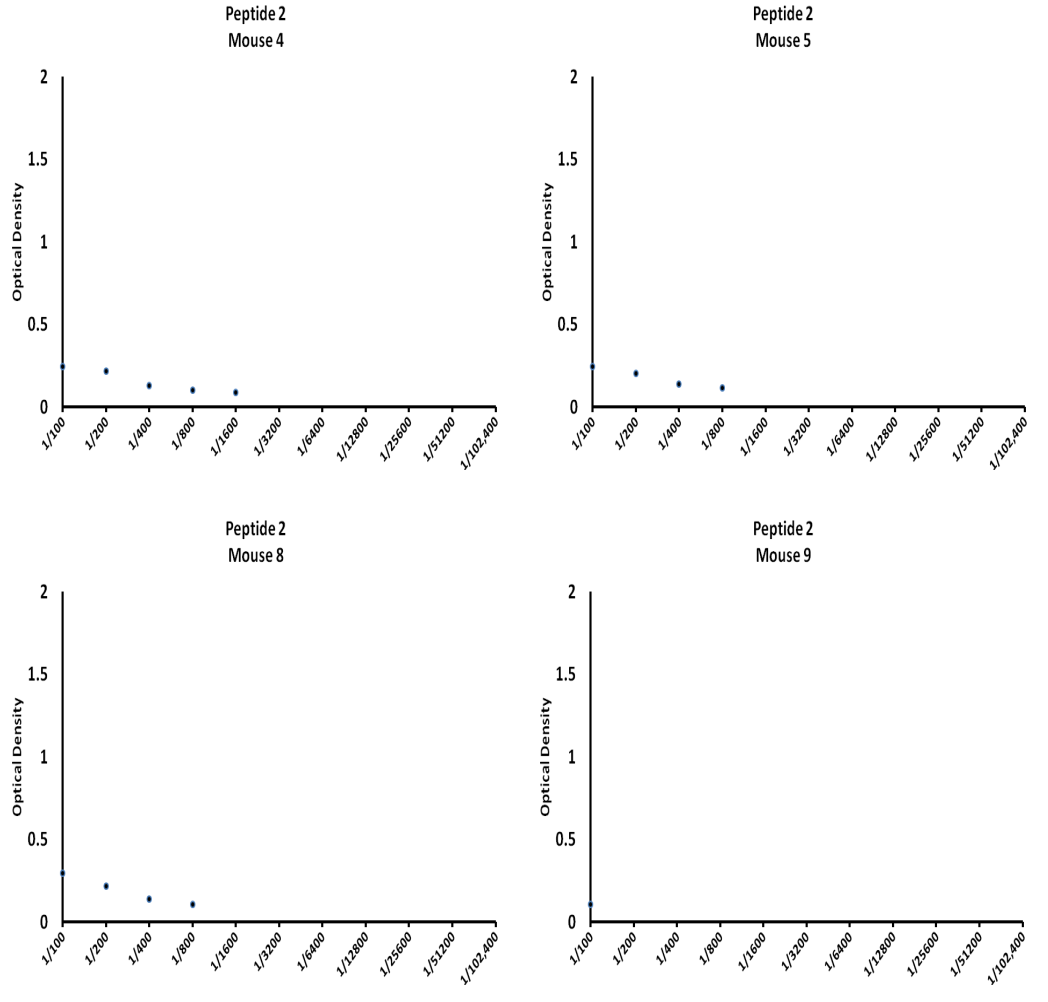


The graph in the box shows the highest titre was obtained from mouse 2 (M2)'s serum. Mice were immunised thrice with Peptide 1. Peptide 1 corresponds to ω-gliadin/C-hordein peptide.

The serum titre of M2 was 1/25600. M2 was given an additional IV booster containing 50μg of immunogen without adjuvant. All mice were given the same

concentration of immunogen in the first and second dose (300 µg). However, the concentration of the third dose was increased to 1000 µg/ml in Mouse 2 (M2) and Mouse 3 (M3) only.

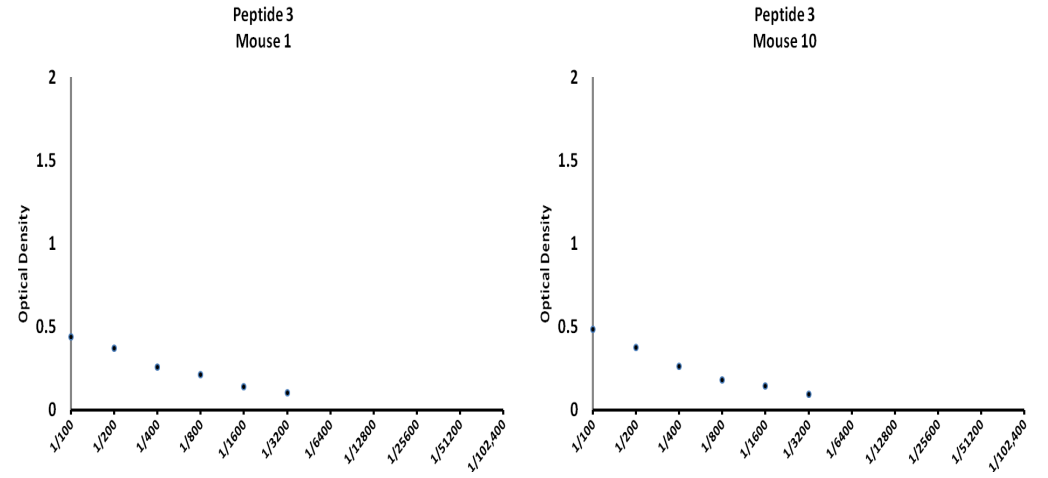
Figure 4.4: IgG titre of Peptide 2, immunised mice sera on a plate coated with 25µg/ml PTG

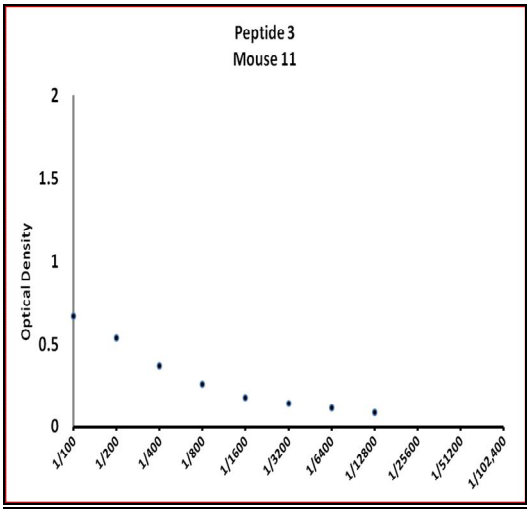


Mice were immunised thrice with Peptide 2. Peptide 2 corresponds to rye secalin derived peptide. None of the immunisations were successful. All mice were given the same concentration of immunogen in the first and second dose of

immunisations (300μg). However, the concentration of the third dose was increased to 1000 μg in Mouse 4 (M4) and Mouse 5 (M5) only.

Figure 4.5: IgG titre of Peptide 3, immunised mice sera on a plate coated with 25μg/ml PTG





The graph in the box shows the positive titre obtained from mouse 11 (M11)'s serum. Mice were immunised thrice with Peptide 3. Peptide 3 corresponds to ω-gliadin/C-hordein peptide in native form, where the 9th amino acid is Q instead of

E. The serum titre of M11 was 1/12800. M11 was given an additional IV booster containing 50μg of immunogen without adjuvant.

All mice were given the same concentration of immunogen in the first and second dose of immunisation (300 μg). However, the concentration of the third dose was increased to 1000 μg/ml in all mice, except in M10.

4.5.1.2 Second Attempt

The mice sera were tested with 25μg/ml PTG after the second dose was given. The details of the immunogen were previously explained in Section 4.4.7. The concentration for each dose was explained in Table 4.2. The sera assessment after the second dose is shown in Table 4.4 below. The ELISA detection dilutions after the second assessment are displayed in Figure 4.6, 4.7, 4.8, and 4.9.

Table 4.4: ELISA assessment: the IgG titre of mice sera after immunisation with peptides conjugated to PPD (Second Dose)

Supernatant	Titre
Positive control-PN3	1/102,400
Negative control-BSA	1/100
M12-Peptide 1	1/6400
M13-Peptide 2	1/3200
M14-Peptide 3	1/3200
M15-Peptide 1	1/6400
M16-Peptide 2	1/3200
M17-Peptide 3	1/3200
M18-Peptide 1	1/6400
M19-Peptide 2	1/12800
M20-Peptide 3	1/12800
M21-Peptide 1	1/6400
M22-Peptide 2	**1/102,400**
M23-Peptide 3	**1/25600**

Peptide 1= Omega-gliadin/C-hordein peptide
Peptide 2= Rye-secalin derived peptide
Peptide 3= Omega-gliadin/ C-hordein peptide with Q substitution at 9th position

Two of the highest serum titre mice were chosen for fusion

1) M22- a titre of 1/ 102,400 against 25µg/ml PT gluten
2) M23- a titre of 1/25,600 against 25µg/ml PT gluten

Selected mice (M22 and M23) were previously immunised twice with the lowest concentration of immunogens that was 150µg (See Table 4.2). These mice were then given booster immunisation and culled. However the post-fusion plates (from M22 and M23) were contaminated by fungal infection.

Using the remaining mice (M12-M21), immunisations were re-assessed by ELISA. As some of the mice showed a good titre, the immunisations were not repeated.

The following are the ELISA detection dilution graphs demonstrated after the second ELISA assessments (Figure 4.6, 4.7, 4.8 and 4.9).

Figure 4.6: IgG titre of PN3 MAb (Positive Control) and BSA (Negative Control), on a plate coated with 25μg/ml PTG

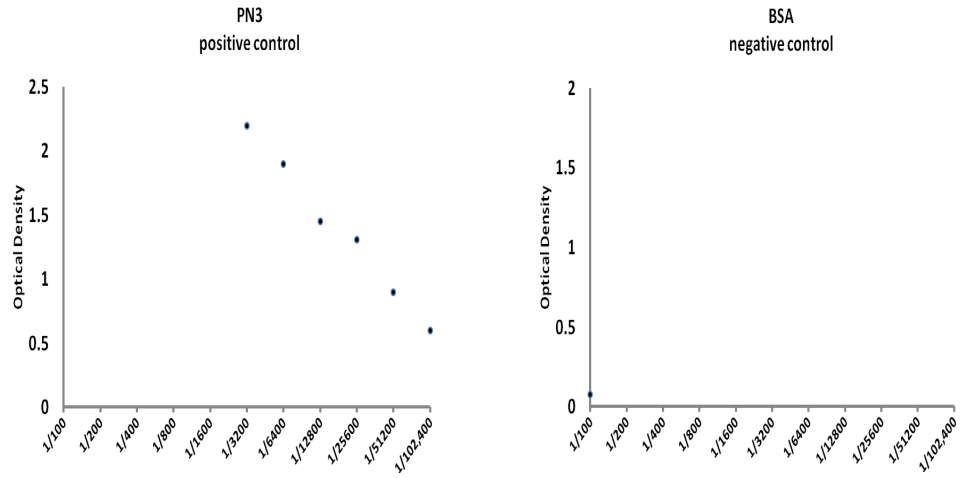
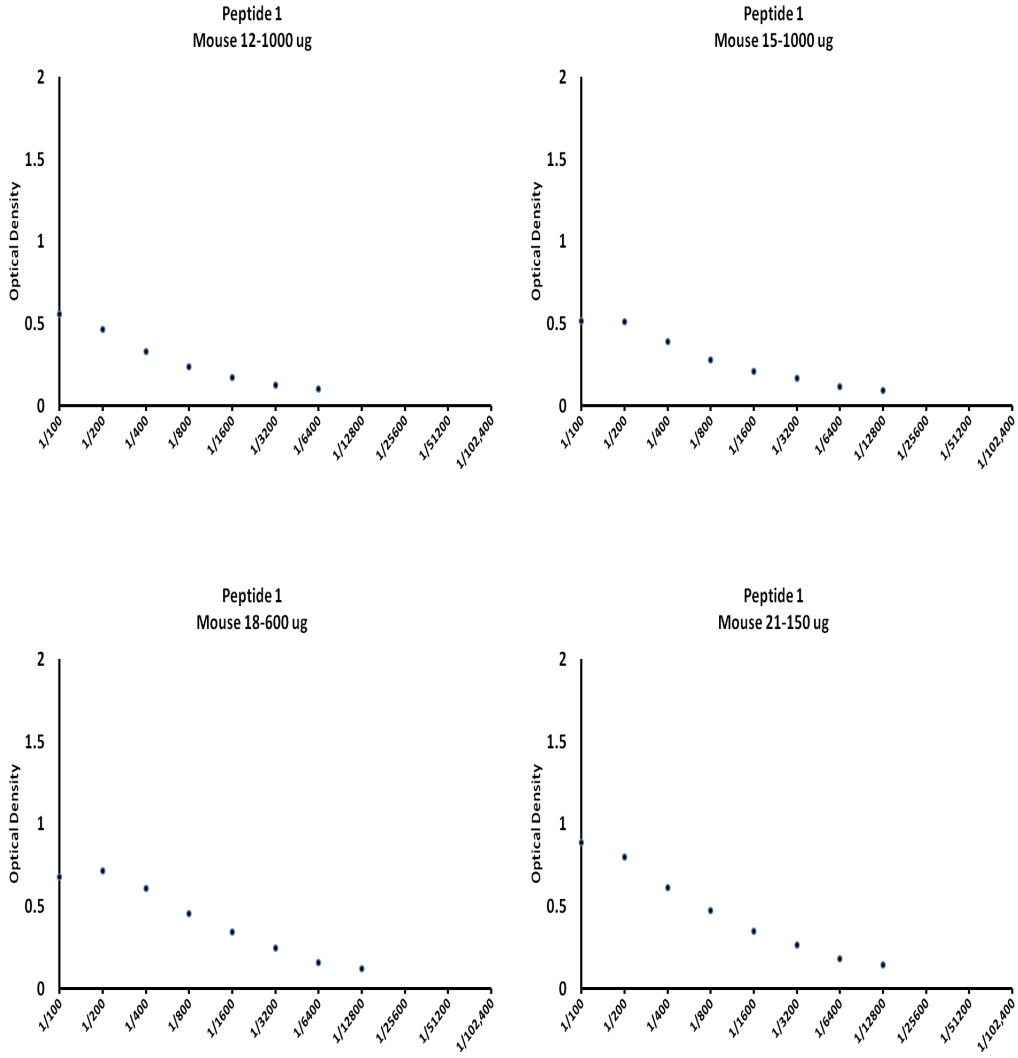


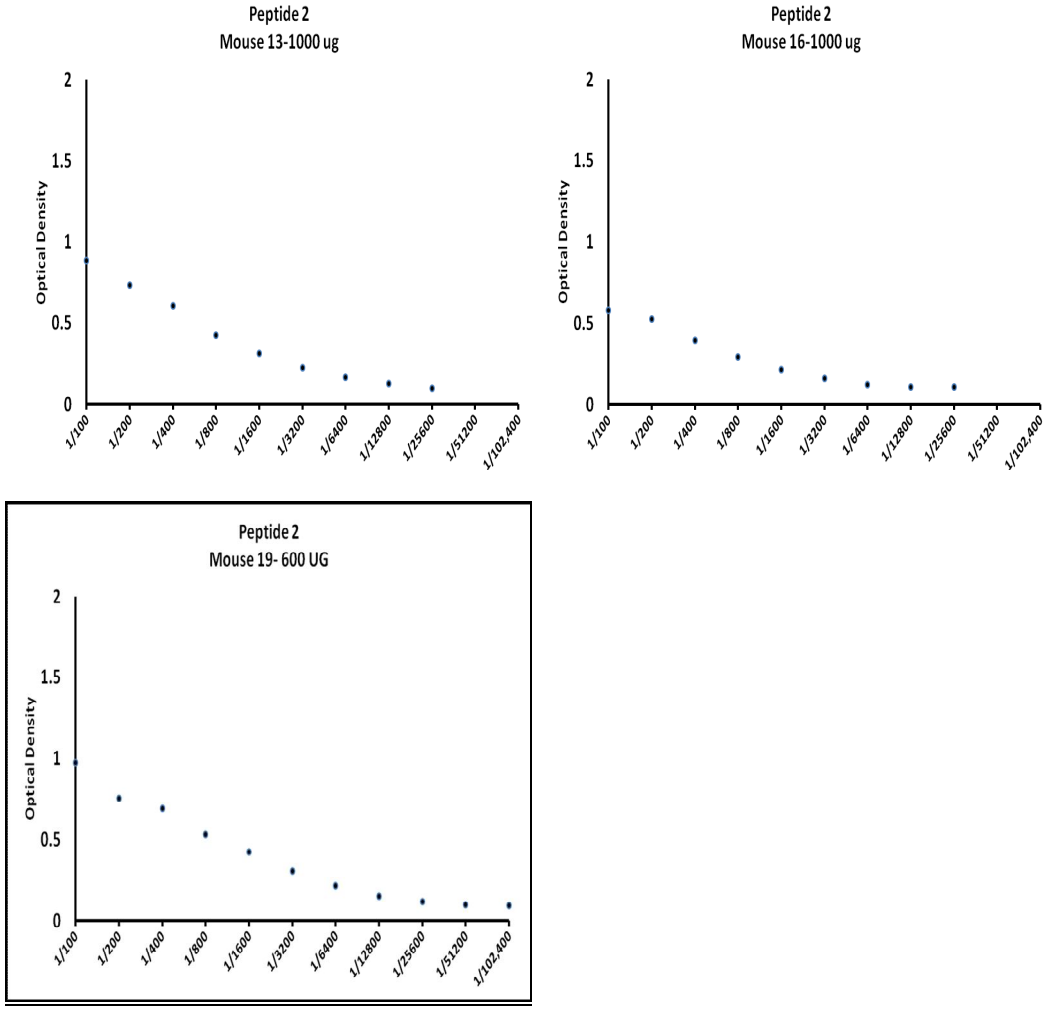
Figure 4.7: IgG titre of Peptide 1, immunized mice sera on plates coated with 25µg/ml PTG



Peptide 1 corresponds to ω-gliadin/C-hordein peptide.

Mice: M12 and M15 were given 1000 µg of immunogen in the first and second dose. M18 received 600 µg and M21 was administered with 150 µg of immunogen, both were during the first and second immunisations. None of the mice were given additional IV booster. They were also not selected for fusion.

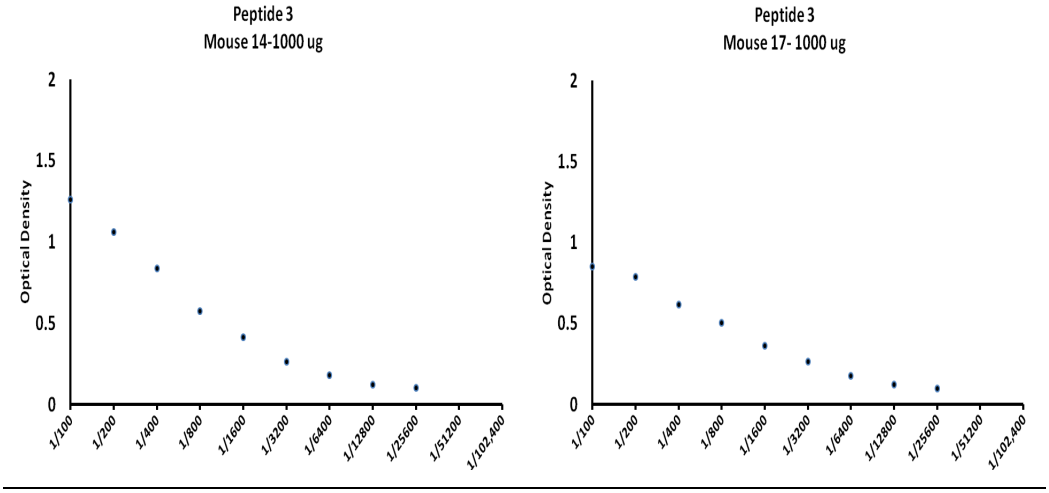
Figure 4.8: Titre of Peptide 2, immunised mice sera on plates coated with 25µg/ml PTG

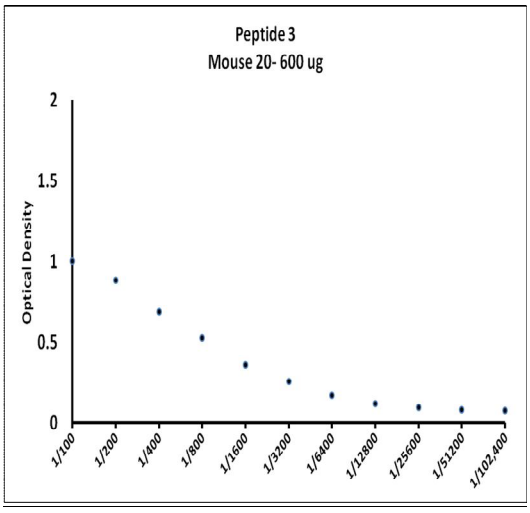


The graph in the box shows the positive titre from M19's serum, and chosen for fusion. Peptide 2 corresponds to rye-secalin derived peptide.

M19 had two immunisations with 600 µg of immunogen. This titre was obtained from the second assessment (no third dose was given). M19 was further given an additional IV booster containing 50 µg of immunogen without adjuvant.M13 and M16 were given the same concentration of immunogen in the first and second immunisations (1000 µg).

Figure 4.9 : Titre of Peptide 3, immunised mice sera on plate coated with 25μg/ml PT gluten





The graph in the box shows the positive titre from M20's serum, and chosen for fusion. Peptide 3 corresponds to ω-gliadin/C-hordein peptide in native form, where the 9th amino acid is Q instead of E.

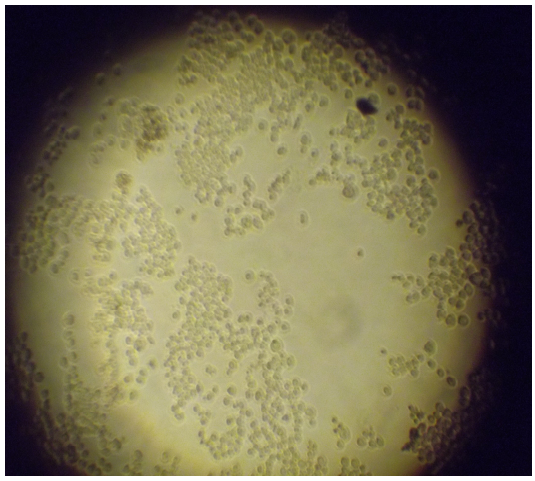
The concentration of immunogen in the first dose is same as the second dose. M20 had twice immunisations with 600 μg of Peptide 3. This mouse was given an

additional IV booster containing 50 μg of immunogen without adjuvant. M14 and M17 were given 1000 μg of immunogens.

4.5.2 Hybridoma formation

From 23 immunised Balb c mice, 6 mice were sacrificed for fusion. Two fusions were undertaken by obtaining splenocytes from first bred mice (M2 and M11). The other four were conducted during the second attempt (M19, M20, M22 and M23). The presence of hybridoma in HAT medium after the death of unfused cells was the first indicator that the fusion was successful and hybridoma formation was promising. Fusions from M11 and M19 did not appear to have hybridoma colonies. Cells obtained from M22 and M23 fusions could not be cultured further, since both plates had been infected with fungus. It was observed that fusions from M2 (first attempt) and M20 (second attempt) might bear hybridoma colonies. However, the hybridoma colonies from M20 were not as healthy as M2. M2's cells suspension of post fusion was aliquoted 1ml/well. From 10 wells, only 5 wells showed potential hybridoma formation. Some had larger colonies at very early stage. Some were observed to contain very few cells and others appeared to have fibro-blast cells and macrophages. Only 2 wells of M20 contained small colonies of cells, presumably hybridoma. Figure 4.10 shows a photograph of hybridoma colonies growing in HT medium.

Figure 4.10: A few large colonies of hybridoma growing in HT medium. This well was screened and selected for cloning.



4.5.3 Secretion of antibodies

The established hybridoma colonies were screened for antibody production. Supernatant from each well was collected and tested for both IgM and IgG anti-peptide antibodies. Although many large colonies were observed in several wells, all did not contain any antibody, except for one well from M2 and one well from M20. Supernatant from both wells contained IgM class of antibody.

Mouse	Immunogen Used	Number of wells containing hybridoma colonies	Positive IgM response	Positive IgG response
M2	Peptide 1	5/10	1/5	No
M20	Peptide 3	2/10	1/2	No

4.5.4 Cloning and MAb production

Although the produced antibody was IgM rather than IgG, the cells were cloned using limiting dilution technique as a prelude of undertaking more monoclonal work in the future. This was done using 96 microtitre plate, with 0.5 cells per well. Cloning was only undertaken during the first attempt since the second attempt also produced IgM antibodies. The positive hybridomas from M2 did not generate large colonies following cloning. Despite the attempt to use fresh splenocytes, the clones did not show any growth enhancement. It is assumed that the clones were no longer viable after 14 days. The attempt at cloning failed, probably due to instability of the cells. In addition, the hybridoma did not produce the antibody of interest, IgG that has longer shelf-life and could be applied for the development of improved immunoassays.

4.6 Discussion

Two attempts at MAb production were made. Mice were maintained on GFD since this leads to enhanced serum immune response to the injected immunogen (Ellis 1998). A GFD was introduced as soon as the breeding pairs arrived. The first generation of offspring was breast-fed from mothers that were on a GFD, and they continued to consume a GFD whilst weaning. This diet will cause the resultant mice to be intolerant to gluten, such that they can then develop antibodies when they are immunised with the respective peptide immunogens.

In earlier work of MAb production by our group, the operator mentioned that the numbers of offspring were reduced when breeding pairs were on a GFD. However in this study, GFD has not shown any effect on the number of litters produced. Fifteen pups were obtained during the first breed, of which 8 were female. The second breed produced 12 pups (6 males, 6 females). Male or female animals could be used for the procedures; however, female mice were reportedly preferable for immunisations due to their placid nature when housed in groups for prolonged times (Liddell and Weeks, 1995). Mice were also inoculated with BCG vaccines before the first dose of immunogen was given to initiate the immune response. The initiation of immunisation should be performed when the animals are of between 8 to 12 weeks old. If the recipients are very young, immunological tolerance is often induced rather than immune response. The efficiency of immune system also less if the recipients are too old (Reeves 2000).

It was thought that shorter interval between immunisations could lead to the production of IgM antibody (Campbell 1984). Hence, in this study, the second or third subcutaneous immunisation was given after four weeks, allowing the mice to produce appropriate immune responses. The mice were checked for

immunisation sufficiency 7 to 10 days after the last immunisations that was the third (first attempt) and second (second attempt).

The tail bleed results have shown that the some mice were adequately immunised (Table 4.4 and Figure 4.3, 4.5, 4.8, and 4.9). The fusions were successfully undertaken at both attempts. This was confirmed by the observed presence of cells, presumed to be hybridoma (Figure 4.10) following the dead appearance of splenocytes and myeloma cell after several days. However, the ELISA screening results of the hybridoma supernatants demonstrated IgM production rather than the desired IgG. The IgM class antibody is known to be relatively unstable so could not be used in kits designed for food screening. The IgG class is the antibody of interest as it has longer shelf life and is compatible with the immunoassay kits for gluten quantification.

It was initially thought that a single booster injection was probably insufficient to stimulate the spleen directly. It has been known that some studies have included several booster injections (Brown 2000) and but others might exclude this booster injection (Spaniej Dekking, 2004). Secondly, it is assumed that the mice were not sufficiently well immunised. The mice might have required additional IFA injections before the booster injection was given (Ciclitira, personal communication). For each additional dose, the response of mice sera should be assessed. This however could not be undertaken due to regulations made by Home Office that only allows the maximum of four injections.

The other explanation for this is probably the interval between the last immunisation with the intravenous booster was prolonged. It was possible that those mice have reverted to produce the primary class of antibody. In the second

attempt, the myeloma cells had been infected with fungal contamination. The culture had to be discarded and new line of myeloma had to be thawed before fusion could be undertaken after three weeks. Therefore, the duration between the last immunisation (second dose) with the IV booster was extended to more than four weeks.

Despite the fact that the produced antibody is the IgM class in the first attempt, the cells were further grown for cloning. In previous experiments taken as a prelude to monoclonal work, the CO_2 independent medium and RPMI medium were compared for the growth of hybridoma. Since the hybridoma appeared to grow better in RPMI, RPMI was used in this study. It is hoped in the future, subsequent monoclonal work will be sought using the same immunogens. If the MAbs to these peptides could be raised, they can be used in combination with other established MAbs by our group, in view of developing a more improved assay for gluten quantification in foods for coeliac patients.

CHAPTER FIVE: DEVELOPMENT OF COCKTAIL COMPETITION ELISA FOR GLUTEN QUANTIFICATION IN STARCHES

5.1 Introduction

The only functional treatment for CD is permanent adherence to a GFD, strictly avoiding foods containing wheat, barley and rye prolamins. Many of the products labelled as gluten free could possibly be contaminated by traces of gluten, or small quantities of gluten remain trapped even after several chemical methods and food processing steps. This raises a question on how safe is the gluten free food for coeliac individuals.

Various methods of gluten quantification have emerged but concentrated on a particular toxic fraction. The safety of foods for coeliac individuals really depends on labelling, thus the exact amount of gluten should be able to be confirmed and standardised. Perhaps, the coeliac consumers can self-check if a simple assay is developed and becomes handy at home.

Several studies to assess the minimum quantity of gluten that could trigger mucosal changes have been undertaken. Professor Ciclitira and colleagues demonstrated the toxicity of gluten fraction with different doses of whole gliadin given to coeliac patients. Measurements were taken before the challenge and after 2, 4 and 6 hours by in-situ biopsy. The 10mg dose had no effect by 6 hours but the 100mg dose induced mild changes to villous architecture and an increase in IEL count by 4 hours. Gliadin doses of 500mg and 1000mg showed moderate and severe villous flattening, respectively (Ciclitira 1984). Coeliac patients appeared to be able to safely consume around 10mg, and 50mg of gluten is

harmful for CD individuals (Catassi 2007). A daily intake of <10-50 mg gluten is unlikely to cause significant histologic abnormalities (Catassi 2007, Catassi 2008)

In 2008, the Codex Alimentarius Committee on Nutrition and Foods for Special Dietary Uses adopted a revised standard of 1) 20 mg/kg for 'gluten-free' labelled products and 2) 100mg/kg for 'very-low gluten' labelled products (Codex Alimentarius 2008). The rules were then introduced as law by the European Union in 2012.

Gluten cannot be easily measured as it comprises gliadin, LMWand HMW glutenin subunits (GS). Many immunostimulatory epitopes lie within gliadin (Arentz-Hansen 2002, Tye-Din 2010), that could be further sub-divided to α-, γ-, and ω- gliadin. Each of the gliadin sub-fractions consists of distinct protein (Shewry and Halford 2002); hence the quantification system should include the total gliadin sub-fractions. Besides gliadin, it is possible that more of the immunostimulatory epitopes could be discovered in the less characterised glutenins, the LMW-GS (Vader 2002a) and HMW-GS that was demonstrated to be highly toxic *in vivo* to CD individuals (Dewar 2006).

There have been several reports of enzyme linked immunoassays to detect and measure gluten in foods. The first known ELISA assay was based on wheat α-gliadin and whole gliadin using polyclonal rabbit or mouse antisera. The assay for the whole gliadin could detect α-, β- and γ- gliadins but not ω-gliadin nor constituents from other toxic prolamins such as barley hordein and rye secalin (Windemann et al 1982, Howdle 1990). The currently available assay kits are based on the R5 antibody (Valdes 2003) that is able to detect all sub-fractions of gliadin and anti-ω-gliadin antibody (Skeritt 1991) that only recognises ω-gliadin.

The former has been adopted by Codex as an official method for gluten measurement. Although these developed assays are generally sensitive, they do not measure glutenin, the other toxic component of gluten (Dewar 2006). The commercially available assay adopted by Codex cannot correctly quantify the actual gluten contamination, since it relies on multipling the gliadin content by the factor 2. This can inaccurately estimate gluten content. Between wheat varieties, there is a difference in the ratio of gliadins to glutenins present. High pressure liquid chromatography has demonstrated that the ratio of glutenin to gliadin varies between 0.2 to 5.8 times (Wieser 2003).

Another complication of gluten protein detection is that of the suitability of the detection system to detect small protein fragments and peptides, which could be present in foods, even if they have undergone chemical processes and been labelled as gluten-free. Moreover, wheat starches and wheat-derived protein hydrolysates are widely used in the food industry, and may contain small CD toxic fragments. Sandwich ELISA can only detect larger fragments. Having these flaws in current commercially available systems, there is a concern for better assay that allows for gluten detection and quantification at very low level. A measurement system should correspond to each and every toxic component in gluten that stimulates T cells response in CD patients. In addition, the system should be able to even detect very small fragment of gluten protein in foods labelled as gluten free.

We proposed to develop an improved assay based on cocktail competition format for gluten quantification which includes the toxic fractions, gliadin and glutenin, and other disease activating epitopes from barley hordein and rye prolamin. The potential advantages of this system are the ability of quantifying small toxic

fraction of peptides and the simultaneous measurement of all toxic components at a time regardless the size of the intact protein. This will allow for gluten quantification in processed foods manufactured for coeliac consumers. If this assay can be developed, a coeliac patient can easily check if the food is confirmed safe for consumption.

The proposed cocktail competition assay will be developed based on combination of murine MAbs, successfully produced by our group. The MAbs used in cocktail competition ELISA were PN3 against 33 mer-gliadin (Ellis 1998), CDC5 raised against 56-75 gliadin peptide (Anderson et al 2000, Arentz-Hansen 2000) and high molecular weight glutenin (CDC7) (Ciclitira 2006a). These MAbs have been extensively use for gluten measurement in our laboratory.

PN3 has previously used to develop a sensitive assay and was able to detect gliadin at 4ng/ml recognising the motif QQQPFP which is found throughout all Triticeae fractions as well as in avenins (Ellis 1998). The antibody CDC5 (Ciclitira 2006b) was raised against immunodominat α-gliadin epitope that also demonstrated *in-vivo* toxicty (Fraser 2003). The cocktail of MAbs will be mixed with the antigen before adding to the pre-coated competing antigen on the ELISA plate. The value obtained from a single and multiple MAbs (combination) is then compared. It is assumed that the cocktail (combination of MAbs) will generate a greater specificity and sensitivity of quantified gluten in foods specialised for individuals with CD.

5.2 Aim

The aim is develop an improved cocktail competition ELISA assay for gluten quantification in purified wheat starches.

5.3 Objectives

The objectives of this study are:

- To determine the most suitable MAbs combinations in cocktail competition ELISA, in a view to develop gluten standard for reference for gluten quantification in starches
- To extract gluten protein and peptides from purified wheat starches
- To optimise the concentration of coating antigen, MAbs, and competing antigen using simple sandwich ELISA
- To quantify the total value of gluten (corresponds to gliadin and glutenin) in wheat starch using the cocktail competition ELISA
- To compare the value of gluten obtained from assays using multiple MAbs (cocktail) with single MAbs.

5.4 Methodology

5.4.1 Preparations of buffers and reagents

All of the buffers used in this experiment were prepared using deionised water from a Mili-Q purification system (Milipore Bedford, USA). The Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) buffer contains 40% (v/v) of Tris-HCl solution (50mM, pH 7.4), 0.1% (w/v) TCEP reducing agent and 60% (v/v) of glycerol as the extractor solvent. The Tris-HCL solution was prepared by dissolving approximately 2.422g of TRIZMA Base (T6066, Sigma), and adding with 2.76ml of 6N HCl (Scharlau, AC0752) in deionised water up to 400ml. The pH was ensured to be 7.4. Sixteen millilitres of Tris-HCL solution was pipetted into a small Schott bottle. Approximately 0.04g of TCEP powder was added and mixed until it was dissolved. Subsequently, 24ml of glycerol was added and continued with further mixing until everything was completely liquefied. The buffer was stored at room temperature.

Carbonate buffer (0.05M, pH 9.6) was prepared by dissolving the contents of one carbonate-bicarbonate buffer capsule (C3041, Sigma) in 100 ml of deionised water and measuring the suitable pH. This buffer was used in the preparation of coating antigen (PTG). We have previously used 60% alcohol to dilute kolibri gliadin to the desired concentration. However the diluent was changed in this study since the antigen are soluble in water based buffers.

PBS-Tween (PBS-T) buffer (0.01M, pH 7.4) was prepared by dissolving 5 PBS tablets (P4417, Sigma) in 1000 ml of deionised water and adding 0.05% (500 µl) of Tween 20 (663684B, VWR International Ltd, Lot 84137915). This buffer was used in washing steps and diluting MAbs to the desired concentration. All steps

required three washes, except in the competition ELISA, the washing step after blocking. Washing was done by thorough rinsing with 250 µl of PBS-T buffer.

Blocking agent was prepared by dissolving 1% of BSA in PBS-T buffer. The mixture was mixed on magnetic stirrer, divided into aliquots, irradiated and stored in -20°C for future use. Prior to undertaking ELISA assays, the blocking agent was placed in the incubator to warm. Blocking agent was used in the assays to block non-specific binding sites after coating; any unbound antigens will be eliminated during washing.

The primary antibodies in these assays were MAbs developed previously by our group. In competition ELISA, the antibodies were prepared just before the competition step by diluting to the desired concentration with PBS-T buffer. In the case of optimising the antibody concentration, MAb was added to the first well of the microtitre plate and serial doubling dilutions were made with PBS-T.

The secondary antibody, antimouse IgG-alkaline phosphatase (A4312, Sigma) was prepared at the concentration of 1 in 2000 with PBS-T. The liquid substrate pNpp (P7998, Sigma) did not require any dilution and added directly after washing.

5.4.2 Preparation of murine MAbs

Murine MAbs against gliadin and glutenins were successfully developed by our group. PN3 (Ellis 1998) is a murine anti-peptide monoclonal raised against a 19-amino-acid A-gliadin peptide, known to cause an innate immunological responses in the small intestinal mucosa of coeliac patients (Sturgess et al. 1994). CDC5 was developed to an α-2-gliadin peptide amino acids 56-75 (Anderson 2000, Arentz-Hansen 2000) and cross reacts with barley and rye. CDC7 was generated against

high molecular weight glutenin-GS 1Dy10 subunits (Ciclitira 2006a). The methods used for the production of MAbs were similar to the one described in Chapter Four.

5.4.3 Preparation of antigen

Coating antigen is one of the main components in enzyme immunoassays. Gluten was chosen in this study since it contains both gliadin and glutenin. However the choice of gluten varied. Three types of gluten were taken into consideration; 1) Gluten extracted with TCEP buffer, 2) gluten digested with enzyme pepsin and trypsin (PTG) 3) digested PT gluten but further dialysed and desalted. TCEP extracted gluten could not be measured by bicinchoninic acid (BCA) Protein Assay Reagent for protein concentration because of the presence interfering substance (glycerol) in the buffer; hence, the concentration of the coating antigen could not be furthered optimised. The second type of gluten (PTG) had already been prepared in the earlier studies and described in detail in Chapter Three. The third type of gluten might contain higher purity since salts would be eliminated from the sample.

The dialysis of PTG was undertaken with Spectra/ POR cellulose ester sterile Dispodialyzer (Z36,837-7, Sigma), according to the manufacturer's instructions. Prior to transferring the sample into the dispodialyzer, the 0.1% sodium azide preservative solution was removed and small amount of deionised water was dispensed into the membrane to rinse. In this experiment, the operator has inserted only 1ml of the PTG sample assuming that some of the dialysate might diffuse in during dialysis and the volume would expand. The dispodialyzer membrane was allowed to soak vertically and dialysed against deionised water (dialysate) for 45 minutes. After the dialyses, the sample appeared to have

precipitated and settled mostly at the bottom. Protein quantification was attempted to compare the dialysed and non-dialysed PTG. However, the results of the dialysed PTG were doubtful since the precipitates did not dissolve in the solution.

The non-dialysed PTG was finally chosen and used in all ELISA assays undertaken in this study. PTG was dissolved in deionised water and analysed for protein concentration using BCA Protein Assay Kit (Novagen), against BSA as standard. The BCA assay is based on the detection of copper ions Cu+1 reduced from Cu2+ in an alkaline condition. However, this kit cannot be employed if the protein solution contains some incompatible substances such as glycerol and lipid.

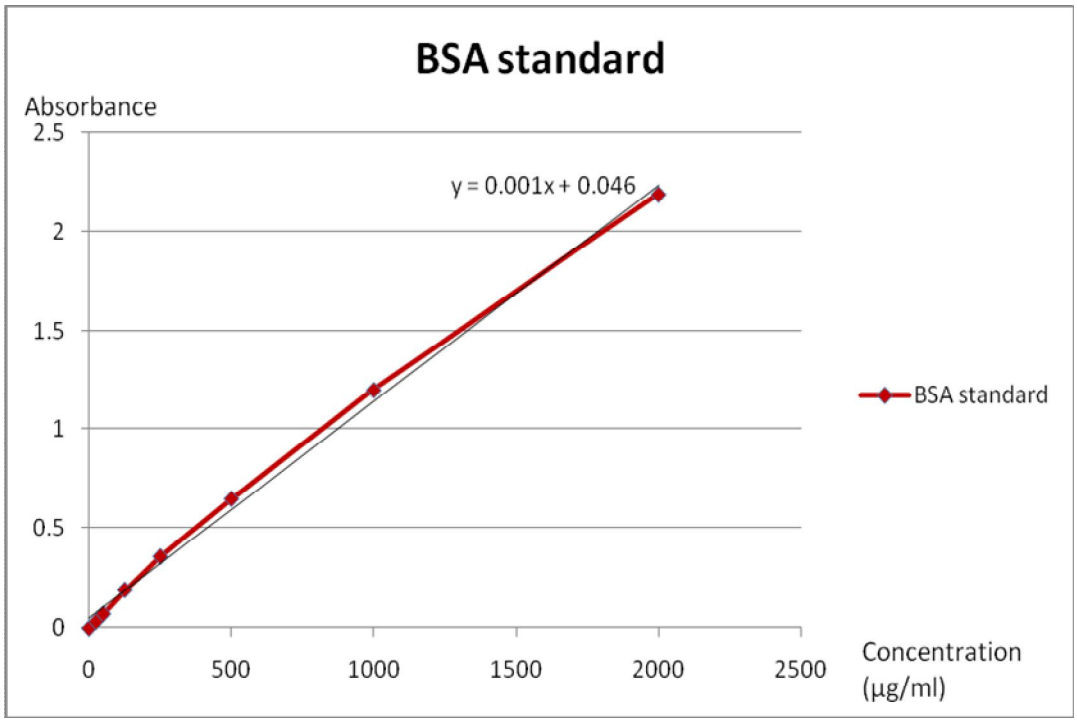
Before the protein concentration of PTG was determined, BSA standard was prepared according to the manufacturer guidelines. A BSA standard of 2mg/ml was provided in the kit and was diluted with deionised water to the final concentration of 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 25µg/ml and 0µg/ml. Deionised water was used as the diluents since the same buffer was used to solubilise the PTG.

BCA working reagent was prepared by mixing 50 parts of BCA solution with 1 part of 4% Culpric Sulfate. The sample was diluted to multiple concentrations with deionised water and prepared in triplicates. Protein measurement was carried out in micro-scale assay that required only 25µl of protein sample in each well and measurement could be conveniently done on a 96-well plate at the same time. Twenty five microlitres the BSA standard or protein sample (PTG) was pipetted triplicate into individual wells of a 96-well late. Approximately 200µl of

BCA working reagent was added to each well. The plates were covered with parafilm and shaken on plate shaker for 30 seconds.

Next, the plates were incubated at 37°C for 30 minutes. The plates were allowed to cool at room temperature before reading at 562 nm on Spectramax Plus (P02359, Molecular Devices) reader. The correct absorbances were obtained by subtracting the absorbances of the blank standard (water only) from the absorbance of measured standard or protein samples. The corrected absorbances were plotted against the concentration of BSA standard to generate the standard curve, as shown in Figure 5.4.3

Figure 5.4.3: BCA standard curve reference for protein measurement in PTG



5.4.4 Extraction of purified wheat starches

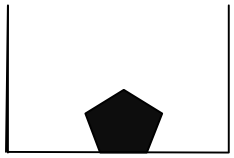
The purified wheat starches A, B and C were obtained from Transia Gluten Test Kit, namely starch A (less than 100mg/kg gluten content), wheat starch B (300-600mg/kg gluten content) and wheat starch C (1000-2500mg/kg gluten content). Each starch was extracted individually. Approximately 100mg of starch was weighed into 1.5ml of Eppendorf tube and added with 1ml of TCEP buffer. The starch was mixed with a small spatula to avoid it from sticking to the walls of the tube. In this study, 12 tubes were prepared at a time equivalent to approximately 1200mg of starches in 12 ml of TCEP buffer. The tubes were vortexed for 30 seconds to increase the homogeneity and inverted occasionally. Next, the tubes were placed in pre-heated water at 85°C for 8 minutes. At this stage, gloves and goggles should be worn since some the tubes might pop out. The tubes were allowed to cool down before being centrifuged (micro-centrifuge) for 3 minutes at maximum speed 13000rpm at room temperature. The supernatants were carefully collected from each tube using a micropipette and transferred into new low-binding protein tube for long-term storage. The extracted starches were irradiated before storing in the fridge.

5.4.5 Optimisation of reagents concentrations

Prior to performing competition ELISA assays, the concentrations of coating antigen and working primary antibodies (MAbs) were optimised by checkerboard ELISA titrating assays. The steps of this direct ELISA is illustrated Figure 5.4.5 below. The optimisation of coating antigen was started with the highest concentration of 50 µg/ml followed by lower concentrations of 25 µg/ml to 0.00625 µg/ml. The plates were coated with 50 µl/well of prepared antigen, and left at 4°C for 18 hours. The antigen solution was discarded and wells washed

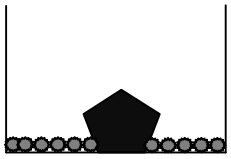
thrice with 250µl of PBS-T. The next step was the addition of blocking agent (the preparation is described in details in Section 5.2.1) to block the unbound sites, 50 µl of the blocking agent was pipette into each well and incubated at 37°C for 1 hour. The plates were washed thrice before adding 50 µl of each of the MAbs, separately. MAb was initially prepared at 1/10 and added in the first well of each row (pre-coated with different concentration of coating antigen, explained above) and serially diluted with PBS-T across the columns of the plate (concentrations reduced to 1/20 to 1/2560). The last well contained PBS only, to act as background. The plates were incubated at 37°C for 1 hour. Again, the plates were washed thrice before adding 50 µl of secondary, detection antibody, anti-mouse IgG alkaline phosphatase (A4312, Sigma), prepared by diluting to 1 in 2000 with PBS-T. Following the 45 minutes incubation at 37°C, the plates were washed and added with 50 µl/well of substrate pNpp. The plates were left in the dark, at room temperature for 40 minutes, to allow colour formation. The plates were finally read at 405nm using Microtek plate reader.

Figure 5.4.5: Steps in indirect ELISA

1) 

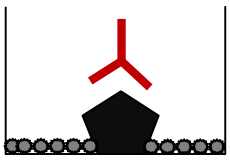
Coat antigen to plate, at 4C overnight

Wash three times with 250 µl of PBS-T

2) 

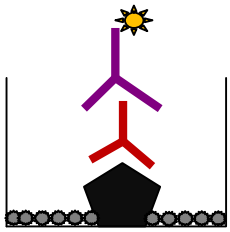
Block the unbound sites with blocking agent

Wash three times with 250 µl of PBS-T

3) 

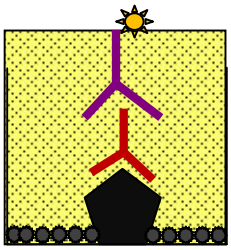
Add primary antibody
(MAbs **PN3** or **CDC5** or **CDC7)**

Wash three times with 250 µl of PBS-T

4) 

Add enzyme-labelled anti-imunoglobulin
(Secondary antibody-detection antibody)

Wash three times with 250 µl of PBS-T

5) 

Add enzyme substrate, colour development could be observed after incubation

CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit, PN3 is MAb raised against 19aa A-gliadin peptide

From the observed absorbance result, the optimum concentration of coating antigen is based on the signals shown even at very low concentration of MAb. Two of the lowest concentrations of the coating antigen were chosen for further assessment. The concentrations of MAbs were further optimised by repeating the assays with the chosen concentration of coating antigens. The optimum concentration of MAbs were selected and reassessed with competition ELISA.

5.4.6 Generating gluten standard curve for quantitative competition ELISA

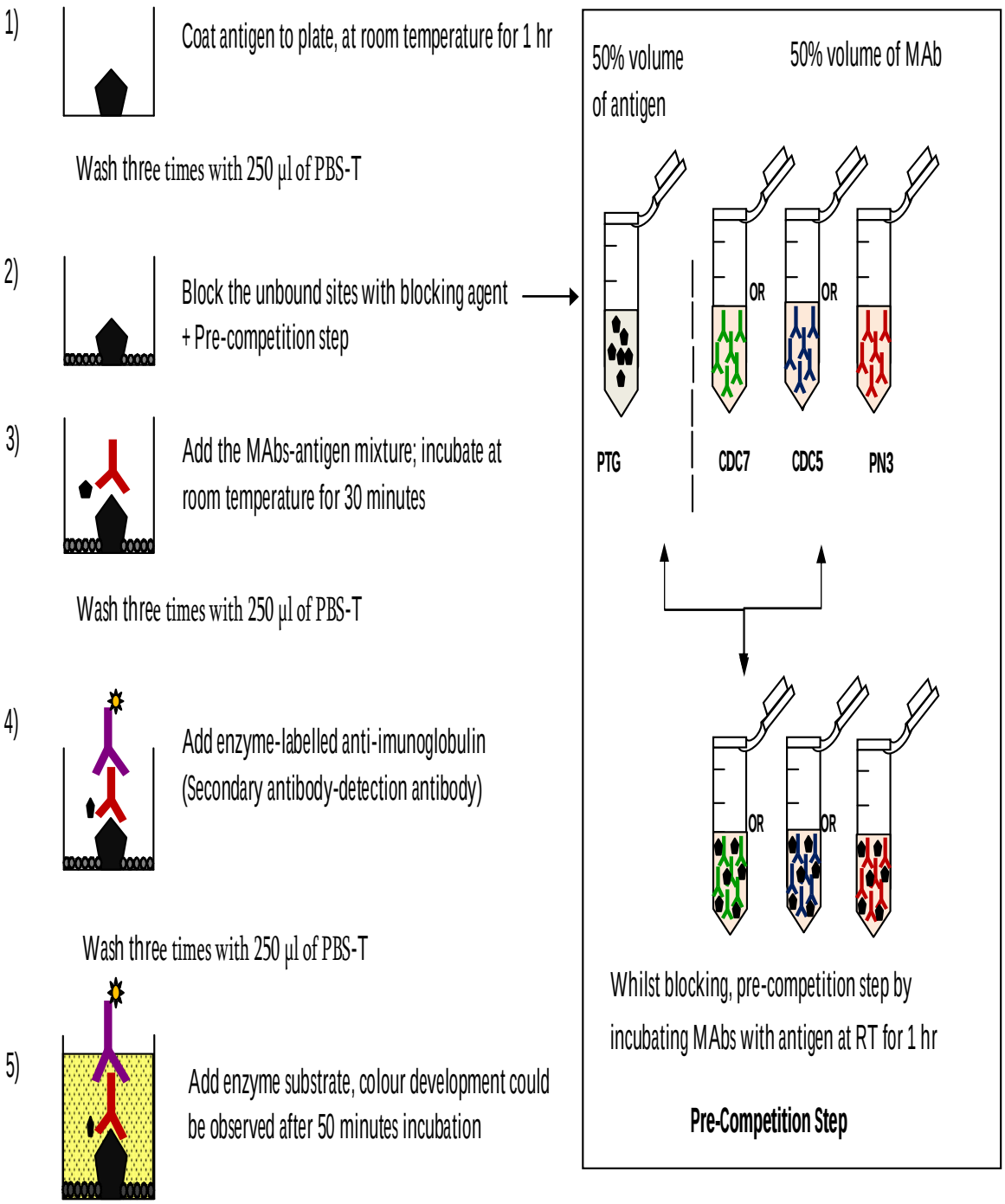
Before a gluten standard curve was generated, competition ELISA assays were continued to choose the most suitable concentration of coating antigen, working primary antibodies and competing antigen. The selections of concentration were initially narrowed down in the direct ELISA assays as described in Section 5.4.5. In the competition ELISA assays, assessments were primarily undertaken on both chosen coating antigens (1.5µg/ml and 2.5µg/ml).

The concentration of MAbs varied. For example, PN3 antibody was best used at 1/160 whereas CDC7 was prepared 1/20. In addition, several experiments were undertaken to optimise the concentration of competing PTG antigen. The concentration was started with highest concentration of 2000 µg/ml and serially diluted to half of the concentration with PBS. After successive of experiments, the working concentrations were chosen in the range of 100µg/ml to 0.0976µg/ml, in doubling dilutions. The plates were coated with the PTG at room temperature for 1 hour. The concentration of the coating antigen were finalised to be 1.5µg/ml in all competition ELISA assays undertaken. The volume of all reagents and samples were 50 µl, unless otherwise stated. Each step required thrice washing with 250 µl of PBS-T except after blocking. The plates were blocked with blocking agent at 37°C for 1 hour, and the contents were removed

by hard shaking. Whist blocking, the pre-competition steps were undertaken by mixing equal volumes of MAbs and working antigen in micro-centrifuge tubes, incubated at room temperature for 1 hour. MAbs were prepared at twice the determined concentration. For instance, if the MAb optimal concentration was 1/160 in direct ELISA assays, the concentration of the antibody would be increased to 1/80 (twice the concentration) in competition ELISA. During the pre-competition steps, the concentration of the MAbs would be diluted half by the presence of the competing antigen, which was also prepared double the required concentration. In this case, the starting concentration of competing antigen was 200μg/ml, so that it would be diluted to 100μg/ml (desired starting concentration) when the same volume of MAbs was added.

After blocking, the MAbs and antigen mixture were added to the wells and the competition step was allowed at room temperature for 30 minutes. After washing, the detection secondary antibody (1/2000) was added and the plates were incubated at 37°C for 45 minutes. Finally substrate pNpp was added and the plate left in the dark, room temperature for 30 minutes. The plates were read at 405 nm with the Mikrotek plate reader. The gluten standard curve was generated by plotting the observed absorbance reading against the concentrations of competing antigen. The simplified steps of competition ELISA is illustrated in Figure 5.4.6 below.

Figure 5.4.6: Steps in competition ELISA



5.4.7 Generating gluten standard curve in cocktail competition ELISA

The steps in cocktail competition ELISA were similar to competition ELISA, as explained in detail above (Section 5.4.6). However, during the pre-competition steps, two MAbs were combined before adding the antigen. The concentration of each prepared MAb should be 4 times more than the concentration used in the direct ELISA. For instance, if the MAb was optimal at 1/160 in the direct ELISA assays, the concentration of the MAb would be increased to 1/40 (4x concentration) in the competition ELISA. During the pre-competition steps, the concentration of the individual MAb would be diluted half by the presence of the other MAb and further half diluted with the presence of antigen. The simplified steps of competition ELISA (Cocktail format) is illustrated in Figure 5.4.7 below.

Figure 5.4.7: Steps in competition ELISA (Cocktail Format)

Similar steps of coating and blocking, except in cocktail competition, 2 MAbs were added to the antigen mixture.

Add the MAbs-antigen mixture; incubate at room temperature for 30 minutes

Wash three times with 250 µl of PBS-T

Add secondary enzyme-labelled anti-immunoglobulin (detection antibody)

Wash three times with 250 µl of PBS-T

Add enzyme substrate. Colour development could be observed after 50 minutes incubation

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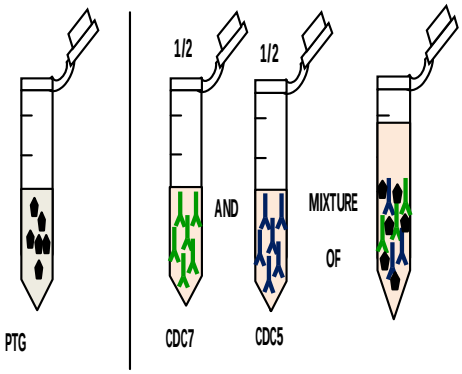
Whilst blocking, pre-competition step was carried out by incubating MAbs with antigen (PTG) at RT for 1 hour,for an example; CDC5 MAb + CDC7 MAb added to the antigen



The summary of the used concentrations in direct, competition and cocktail competition ELISA is simplified in the Table 5.4.7 below.

Table 5.4.7: Summary concentrations of MAbs in different ELISA format

MAb	Direct ELISA	Competition ELISA	Cocktail Competition ELISA
			Two MAbs combined
PN3	1/160	1/80	1/40
CDC5	1/40	1/20	1/10
CDC7	1/50	1/25	1/12.5

CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit, PN3 is MAb raised against 19aa A-gliadin peptide

5.4.8 Quantification of gluten in purified wheat starches

Single competition and cocktail competition ELISA assays were undertaken simultaneously on the same plate to quantify gluten in all types of starches. Basically each assay consists of gluten standard for reference, individual or combined MAbs and starches as samples to be analysed. The methods of preparing the assays were described comprehensively in both Sections 5.4.6 and 5.4.7. Starches were extracted at 100mg/ml, irradiated and kept in at 4◦C for longer storage. Initial concentration in all assays is 100mg/ml that was the absolute starch solution without any dilution.

Then, the starch was serially double diluted PBS to obtain concentration of 50 mg/ml, 25 mg/ml…0.0975 mg/ml. The plates were coated with 1.5 μg/ml of PTG, left at room temperature for 1 hour. Next, the plates were added with blocking

agent, incubated for 1 hour then added with MAb-antigen mixture. The example of plate is shown in Figure 5.4.8 where CDC7 and CDC5 Mabs were combined.

Figure 5.4.8: An example of a plate for gluten quantification using CDC5 and CDC7 MAbs

	1	2	3	4	5	6	7	8	9	10	11	12	
A	PTG= 100 µg/ml	PTG= 50 µg/ml	PTG= 25 µg/ml	PTG= 12.5 µg/ml	PTG= 6.25 µg/ml	PTG= 3.125 µg/ml	PTG= 1.5625 µg/ml	PTG= 0.78125 µg/ml	PTG= 0.3906 µg/ml	PTG= 0.1953 µg/ml	PTG= 0.0976 µg/ml	Blank	CDC7
B	Starch= 50 mg/ml	Starch= 25 mg/ml	Starch= 12.5 mg/ml	Starch= 6.25 mg/ml	Starch= 3.125 mg/ml	Starch= 1.5625 mg/ml	Starch= 0.78125 mg/ml	Starch = 0.3906 mg/ml	Starch= 0.1953 mg/ml	Starch= 0.0976 mg/ml	Starch= 0.0488 mg/ml	Blank	CDC7
C	PTG= 100 µg/ml	PTG= 50 µg/ml	PTG= 25 µg/ml	PTG= 12.5 µg/ml	PTG= 6.25 µg/ml	PTG= 3.125 µg/ml	PTG= 1.5625 µg/ml	PTG= 0.78125 µg/ml	PTG= 0.3906 µg/ml	PTG= 0.1953 µg/ml	PTG= 0.0976 µg/ml	Blank	CDC5
D	Starch= 50 mg/ml	Starch= 25 mg/ml	Starch= 12.5 mg/ml	Starch= 6.25 mg/ml	Starch= 3.125 mg/ml	Starch= 1.5625 mg/ml	Starch= 0.78125 mg/ml	Starch = 0.3906 mg/ml	Starch= 0.1953 mg/ml	Starch= 0.0976 mg/ml	Starch= 0.0488 mg/ml	Blank	CDC5
E	PTG= 100 µg/ml	PTG= 50 µg/ml	PTG= 25 µg/ml	PTG= 12.5 µg/ml	PTG= 6.25 µg/ml	PTG= 3.125 µg/ml	PTG= 1.5625 µg/ml	PTG= 0.78125 µg/ml	PTG= 0.3906 µg/ml	PTG= 0.1953 µg/ml	PTG= 0.0976 µg/ml	Blank	CDC7+ CDC5
F	Starch= 50 mg/ml	Starch= 25 mg/ml	Starch= 12.5 mg/ml	Starch= 6.25 mg/ml	Starch= 3.125 mg/ml	Starch= 1.5625 mg/ml	Starch= 0.78125 mg/ml	Starch = 0.3906 mg/ml	Starch= 0.1953 mg/ml	Starch= 0.0976 mg/ml	Starch= 0.0488 mg/ml	Blank	CDC7+ CDC5
G													
H													

Cocktail Competition

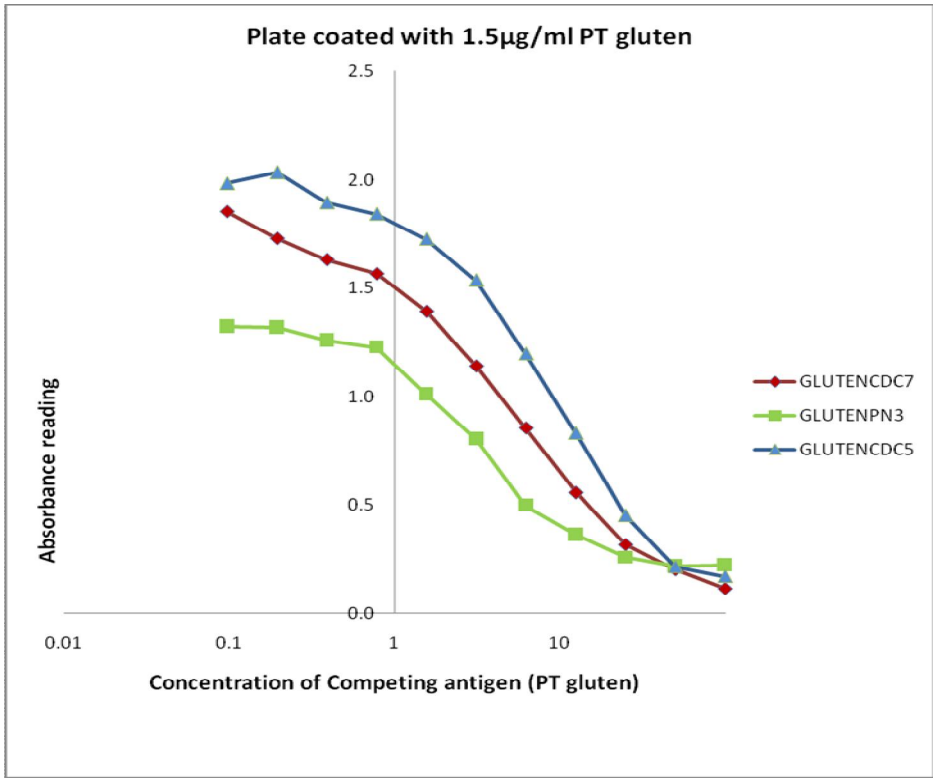
CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit

5.5 Results

5.5.1 Coating antigen

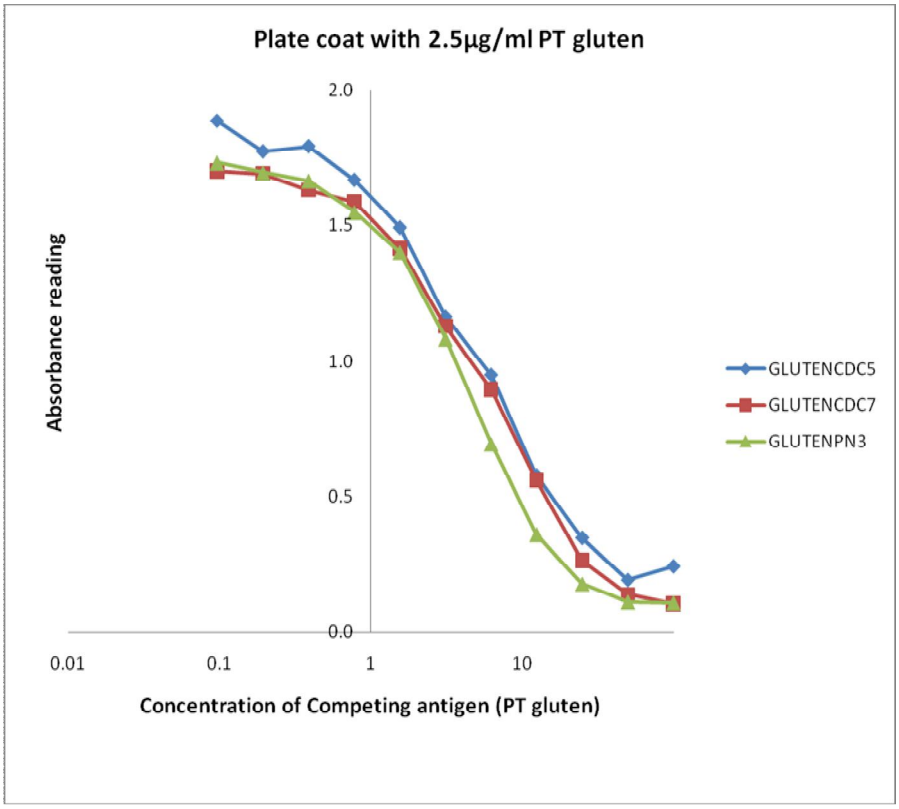
The checkerboard titration revealed the optimal conditions for assays based on use of PTG as coating and competing antigen. For single competition ELISA, the best concentrations for coating antigen were 1.5μg/ml (Figure 5.5.1.1) and 2.5μg/ml (Figure 5.5.1.2).

Figure 5.5.1.1: Competition ELISA on a plate coated with 1.5μg/ml PTG



CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit, PN3 is MAb raised against 19aa A-gliadin peptide

Figure 5.5.1.2: Competition ELISA on a plate coated with 2.5μg/ml PTG



CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit, PN3 is MAb raised against 19aa A-gliadin peptide

In cocktail competition ELISA (combined 2 MAbs), the most optimum concentration was 1.5μg/ml (Figure 5.5.1.3)

5.5.2 Working concentration of competing antigen

The working concentration of PTG antigen was between 100μg/ml to 0.195μg/ml (Figure 5.5.1.3).

5.5.3 Working concentration of MAbs

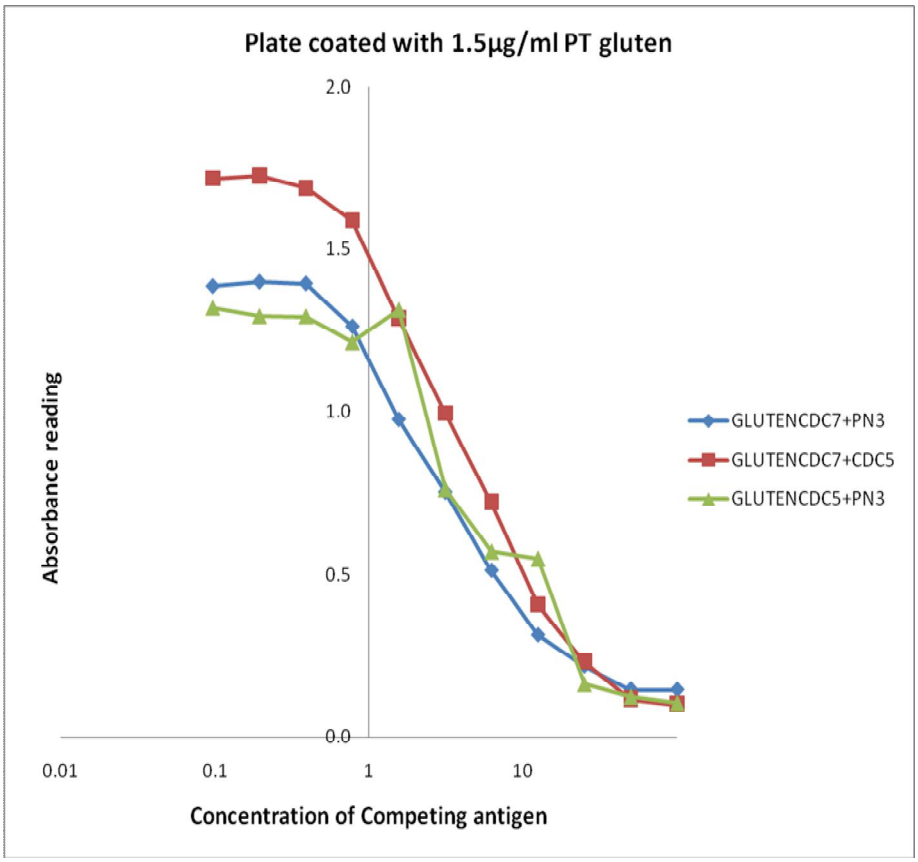
MAbs labelled as PN3, CDC5 and CDC7 were precipitated from the stored hybridoma supernatant. The best dilution for MAbs to be used in pre-incubation step for cocktail competition ELISA (Figure 5.5.1.3) is as follows:

PN3: 1/40

CDC5: 1/10

CDC7: 1/12.5

Figure 5.5.1.3: Competition ELISA on a plate coated with 1.5μg/ml PTG



CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit, PN3 is MAb raised against 19aa A-gliadin peptide

5.5.4 Real sample analysis

Standard solution of gluten over a range of 0.195-100μg/ml was assayed on all plates. The amount of gluten present in each wheat starch was calculated by comparing the curve of starch parallel to the curve of gluten standard. From that, the concentration of gliadin and glutenin in starch could be calculated.

The combination of MAbs for cocktail competition ELISA format is as follows:

1) CDC5 plus PN3
2) CDC7 plus PN3
3) CDC7 plus CDC5

The first combination was undertaken during a preliminary study and subsequently excluded since the assay quantified gliadin only, thus the total gluten content was not obtained (Table 5.5.5.1). Amidon C has been shown to contain the highest amount of gliadin in any cocktail competition ELISA assays. The lowest gliadin content was observed from starch Amidon A that was slightly over the recommended gluten-free label of 20mg/kg, which would increase when glutenin is taken into consideration.

Table 5.5.5.1: Gluten concentration in wheat starches using MAbs CDC5+PN3 in Cocktail competition ELISA format

Wheat starch	**Individual** CDC5 MAb	**Individual** PN3 MAb	Cocktail CDC5+PN3
	Gliadin (mg/kg)	Gliadin (mg/kg)	Gliadin (mg/kg)
Amidon A	21	17	28.8
Amidon B	60	23	43.32
Amidon C	117.9	75	178.6

The next attempt of cocktail competition ELISA assay was to combine CDC7 with PN3, which corresponds to glutenin and gliadin, respectively (Table 5.5.5.2). Although the Amidon A demonstrated the lowest gluten content, but the value exceeded the maximum recommended 20mg/kg. However the result was in agreement with the manufacturer's measurement that is less than 100mg/kg. In addition, the Amidon B demonstrated the slightly higher than the recommended low gluten level (100mg/kg), but was again within the manufacturer's estimation of 100-200mg/kg, when the individual quantification was combined. Amidon C has demonstrated over 100mg/kg of gluten content, although less than the manufacturers estimate of over 1000mg/kg. The value of gluten quantified by the combination of MAbs (Cocktail ELISA) is considerably less than the summation of the two single antibody assays.

Table 5.5.5.2: Gluten concentration in wheat starches using MAbs CDC7+PN3 in Cocktail competition ELISA format

Wheat starch	**Individual** CDC7 MAb	**Individual** PN3 MAb	Summation of Individual quantification (mg/kg)	Cocktail CDC7+PN3
	Glutenin (mg/kg)	Gliadin (mg/kg)		Gluten (mg/kg)
Amidon A	25	33	58	36.7
Amidon B	83	38	121	51.2
Amidon C	180	159	339	114

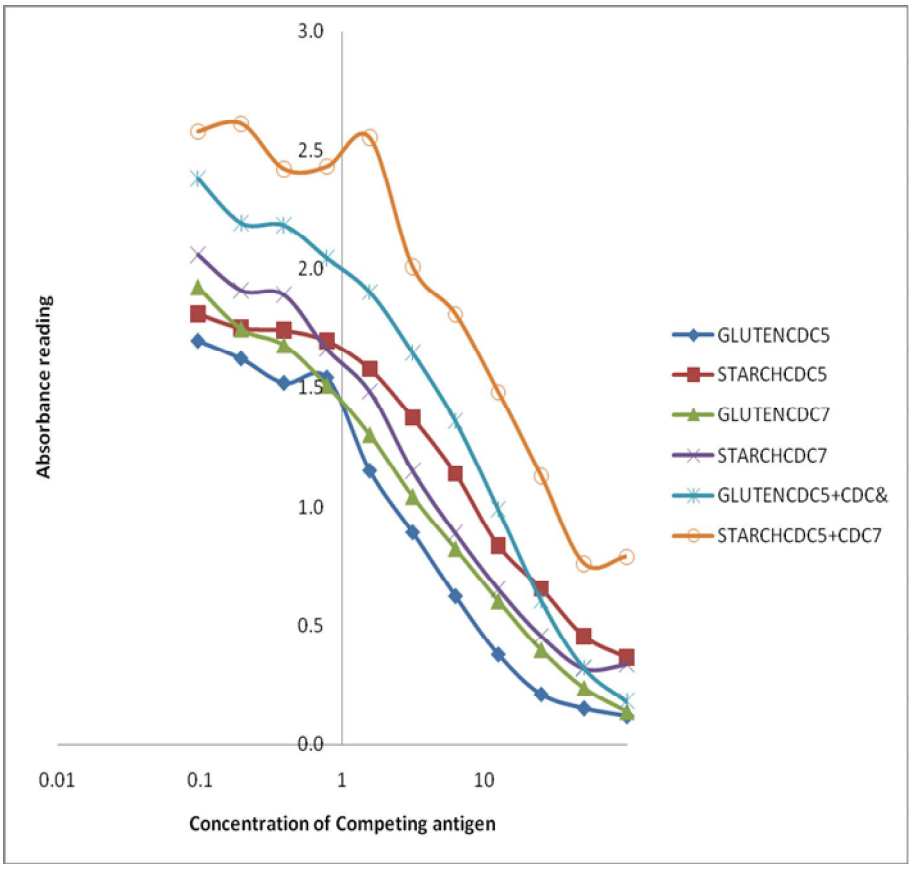
The final cocktail competition ELISA involved combination of CDC5 and CDC7, both cross react with different gluten toxic fractions. The value of quantified protein was more reliable in terms individual quantification, the summation of individual quantification and cocktail format (Table 5.5.5.3).

Table 5.5.5.3: Gluten concentration in wheat starches using MAbs CDC7+CDC5 in Cocktail competition ELISA format

Wheat starch	**Individual** CDC7 MAb	**Individual** CDC5 MAb	Summation of Individual quantification (mg/kg)	Cocktail CDC7+CDC5
	Glutenin (mg/kg)	Gliadin (mg/kg)		Gluten (mg/kg)
Amidon A	16.1	33	49	20
Amidon B	1291	333	1624	484.2
Amidon C	1545	405.67	1950.67	777

The example of gluten standard curve using CDC5+CDC7 combinations to quantify gluten in Starch C is shown in Figure 5.5.5.4.

Figure 5.5.5.4: An example of gluten quantification using CDC5 +CDC7 combination. Gluten was quantified in Starch Amidon C.



CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit, PN3 is MAb raised against 19aa A-gliadin peptide

The first (CDC5 plus PN3) and second (CDC7 plus PN3) combination were finally excluded in this study due several reasons that will be explained in the discussion section. The results for both attempts were shown but not further used in assays for gluten quantification. The third combination (CDC7 plus CDC5) was intended to be used in quantification in other commercially available foods produced for coeliac consumers.

5.6 Discussion

The aim of this work was to develop improved assays for gluten quantification in foods for individuals with CD. In our assays, the standard used for our study was PTG. It was intended for both CDC7 and CDC5 MAbs cross reactivity to give more accurate value of total gluten content. We did however attempt to use TCEP extracted gluten as a standard. However, the concentration of protein in the TCEP extract could not be measured by BCA assays, as the extraction buffer contained glycerol that interfered with the absorbance reading

We have optimised the concentration of coating antigen, competing antigen and MAbs were optimised as previous studies have used different reagents. The pre-incubation times were compared between 30 minutes and 60 minutes, and the latter was finally chosen. The standard curve was prepared using the range between 0.195 and 100μg/ml. The step after coating was blocking in the incubator. Whilst blocking step, MAbs and PTG were pre-incubated together before added to the wells containing coated antigen. This was then followed the addition of secondary antibody alkaline phosphatase.

Two of the major disadvantages of currently available ELISA assay systems are lack of accuracy and unsuitable for detection of small peptides. The currently adopted system randomly estimates the value of gluten by assuming the percentage of glutenin is as much as gliadin, therefore the obtained value of gliadin is multiplied by two. Besides, these methods are based on sandwich ELISA that could only usable for the detection of larger protein fragments. The remaining small peptides could be potentially toxic but simply missed by the current assays.

Our results have shown that all three type of purified starches contained different level of gluten. Concerning wheat starches have been used widely for the basis of GF products, it is important to measure the toxic components to ensure they are suitable for the CD individuals' diets. It was anticipated that wheat Amidon C contains the highest content of gluten followed by Amidon B and Amidon A. The provided Starch C could contain the unacceptable high gluten content.

What is interesting/ peculiar about this data was that, the combination of MAbs in cocktail ELISA did not yield a higher value of gluten compared to quantification using individual Mabs. It was found, on the other hand, the summation of individual quantification could generate higher value, in contrast to what have been predicted. Since gluten comprises both gliadin and glutenin, it was expected that the value gluten should be higher than the value of gliadin alone. Since CDC5 recognises gliadin epitopes only, and CDC7 recognises only HMW glutenin epitopes, epitope masking or steric hinderence could not be the cause of the lower values found in the cocktail assay. Despite of this finding, our assays could still represent as an improved system since both toxic components were measured at once. These improved assays could probably recognise small protein fragments, hence applicable for gluten quantification in commercially available CD specialised foods.

Preliminary experiments were undertaken to compare the MAbs combination that gave the best yield of gluten content in starch. The initial attempt to combine CDC5 with PN3 was found to be irrelevant and unsuitable in this study since that assay could only quantify gliadin, but not glutenin.

The combination of PN3 (anti-gliadin) and CDC7 (anti-HMW-GS) was also taken into consideration. However, the result obtained from the combination of both Mabs, compared to the summation was not as high as the CDC5 with CDC7.

Combination of CDC5 (anti-gliadin) with CDC7 (anti-HMW-GS) yielded the best result for gluten quantification in all purified wheat starches. The CDC7 has been shown to react with wheat, rye and barley by our group (Ciclitira 2006a). As this MAb was raised against HMW-GS, it is possible that the sequence recognised by CDC7 is not distant to rye and barley high molecular weight prolamins, known as HMW secalins, and D hordeins, respectively. The quantification of glutenin using this antibody is not only limited to wheat-derived products or rendered wheat starch gluten free foods, but further screening could possibly be done in foods contaminated with rye and barley. Our results revealed that even the purified wheat starch A contain gluten more than 20mg/kg or 20ppm. This is important to make choice of the source suitable to make foods for the consumption of CD patients.

CDC5 is MAb raised against 56-75 α-gliadin peptide, CDC7 is MAb raised against HMW-GS 1DY10 subunit, PN3 is MAb raised against 19aa A-gliadin peptide

CHAPTER SIX: GENERAL DISCUSSION, CONCLUSION AND FUTURE PROPOSALS

6.1 General Discussion

Individuals with CD need to comply with a GFD as the basis of their treatment. Gluten was previously known as wheat gluten that is comprised of gliadin and glutenin proteins. It is now increasingly accepted as the generic term for CD toxic proteins in wheat, rye and barley; that represent. These proteins contain significant quantities of proline and –glutamine residues. Wheat, the gliadin proteins can be subdivided into α-, γ- and ω-gliadins, while the glutenin proteins can be subdivided into high molecular weight (HMW) and low molecular weight (LMW) subunits. The majority of the coeliac investigations have focused on CD toxic gluten peptides from wheat (Tye din et al 2010, Camarca et al 2009, Dewar 2006, Molberg et al 2003, Vader et al 2002a, Vader et al 2002b, Anderson et al 2000, Arentz-Hansen et al 2000, and van de Wal et al 1998). Studies involving barley and rye are not as numerous (Tye Din et al 2010, Kilmartin et al 2006 and Vader et al 2003).

Wheat α-gliadin-derived peptides have always been thought to be the most CD immunodominant. However, several studies have suggested omega-gliadin to contain potentially more CD toxic gluten peptides (Camarca 2009, Tye-Din 2010). The selected peptides in this study: ω-gliadin/C-hordein and rye peptide (Tye Din 2010) are thought to contain the CD toxic epitopes (Sollid 2012). The nomenclature of CD relevant epitopes has been recently classsified with certain CD toxic motifs listed as unique epitopes because they derive from different cereal species (Sollid 2012). Wheat ω-gliadin/C-hordein comprises of overlapping nine amino acid of DQ2.5-glia-ω1 epitope (Sollid 2012) that is classified as

DQ2-ω-I by different author(Tye din 2010); and DQ2.5-glia-ω2 (Sollid 2012)/DQ2-ω-II (Tye din 2010). The rye-derived peptide lies in the sequences of Sec-α-2 and Sec-α-9, thought to be homologue to DQ2-α-2 and DQ2-α-1 (Vader 2003), respectively.

We have shown that ω-gliadin/C-hordein and rye peptide are CD immunogenic when tested with CD small intestinal gluten sensitive T cell lines. The majority of wheat gluten small intestinal T cell lines cross reacted with these immunogenic peptides. Our study confirms the presence of other important peptides in several Triticae prolamins, other than wheat that could be potentially CD toxic. The author experienced difficulties in establishing CD sensitive T cell lines to barley hordein and rye secalin. However, the author has proven that T cell lines to barley hordein and rye secalin could be generated in addition to wheat gluten protein. These were achieved by co-culturing biopsies from patients with digested prolamins (peptic-tryptic) derived from barley hordein and rye secalin. This is, to our knowledge, the first demonstrations of barley and rye stimulating small intestinal immune responses at an initial stage. Nine barley hordein sensitive T cell lines and 2 rye secalin sensitive T cells were established. Following the success establishing T cell lines to barley and rye prolamins, we suggest these prolamins fractions could initiate future investigation to explore other potentially CD toxic barley and rye peptides.

The *in vitro* study of barley and rye peptides (Chapter Three) has directed us with attempts to produce MAbs to those peptides. The second aim of the thesis is based on the idea that the immunogenic peptides would have the potential to elicit an immune response in mice. This would allow specific MAbs to be developed,

which in turn would serve to improve the sensitivity and specificity of quantifying CD toxic protein in GFD foods to individuals with CD.

Several studies have generated MAbs to CD toxic wheat and rye fractions (Sanchez 2007, Spanej Dekking 2004, Sorell 1998, Ellis 1998, Skerritt 1985 and Ellis 1993). The potential generation of MAbs to ω-gliadin/C-hordein and a rye derived peptide is the first attempts to our knowledge to raise such antibodies. Should the MAbs to these peptides be produced, they could be used in assays that have the potential to detect and quantify additional CD immunogenic peptides in wheat gluten, rye secalin and barley hordein.

We have demonstrated the successful immunisation of mice. However, the resultant antibody of interest could not be produced. It is suggested that this approach could be repeated with higher doses of immunogen. In addition, the intervals between those immunisations should be further optimised. The ELISA screening of sera from immunised mice was undertaken after the second immunisations. The author suggests additional immunisations are required to immunise the mice adequately to generate MAbs. This was previously guided by UK Home Office regulations that reduced the number of immunisations.

The final component of this PhD study was to develop improved assays for gluten quantification in foods (Chapter Five). Using MAbs previously generated by investigators within our group, the student has attempted to combine the MAbs as in cocktail ELISA format. The two main questions were: 1) Will these assays able to quantify glutenin? 2) Will a combination (Cocktail) of different MAbs improve the sensitivity and specificity to quantify CD toxic prolamins in commercially available gluten-free products for individuals with CD?

The answer to the first question is definitely yes since MAb raised to glutenin was employed. This approach is assumed to be more valid than the currently approved that utilised a MAb raised against rye secalin, R5 ELISA. The latter has already been known not to be able to quantify glutenin, that is equally or potentially more CD toxic than wheat gliadin. Despite the ability of the proposed assay to detect glutenin, the combinations of MAbs yielded lower value of gluten compared to gluten measurement individually with a single MAb.

The knowledge of coeliac toxic epitopes, and bio-informatics could be combined to select other potential CD toxic peptides as a prelude to generating commercially available gluten-free foods. We suggest it is possible that many coeliac toxic peptides could be used to develop MAbs with a wide reactivity to CD toxic epitopes.

6.2 Conclusion

The aims of this study were to investigate the CD immunogenicity of barley an ω-gliadin/C-hordein peptide and a rye derived peptide, to produce MAbs to these peptides and to develop an ELISA in the cocktail competition format for gluten quantification in foods for individuals with CD.

We established CD sensitive T cell lines to wheat gluten (n=14), barley hordein (n=9) and rye secalin (n=2). The majority of the gluten sensitive T cell lines demonstrated cross reactivity to ω-gliadin/C-hordein peptide and rye peptide. This suggests that these peptides have CD immunogenicity and potentially *in vivo* CD toxic. These peptides could also be present in all Triticeae prolamins. In the MAb production to the described peptides, successful murine immunisations

were demonstrated with various amount of immunogen that was PPD-conjugated peptides. The resultant antibody was rather IgM than IgG, that is relatively unstable thus unsuitable to be used in ELISA assays for gluten quantification.

Subsequently, the development of Cocktail ELISA was assumed to be competent of measuring toxic proteins, gliadin and glutenin. However, the combinations of MAbs, previously developed by our research group appeared not to have improved sensitivity. The aims of the project were to be successful although they do not fulfil the desired targets. The results of these studies can be considered to be fundamental for undertaking further related work. An example method of the cocktail ELISA development (Chapter Five) for instance, can be used as a preliminary study for further future work.

6.3 Future proposals

This study has demonstrated the CD immunogenicity of ω-gliadin/C-hordein and rye-derived peptide in small intestinal gluten sensitive T cell line. It would be interesting to know if these peptides could cause morphological changes in small intestinal biopsies of CD patients that could be assessed with a CD duodenal organ culture biopsy technique. This would complement the current data. The attempt to develop MAbs resulted in the production of IgM rather than IgG antibody. It is anticipated that in the future, subsequent generation of MAb to CD toxic prolamins could be undertake using the same immunogens. Increasing the number of immunisations is another option to generate IgG class of antibody. These additional procedures have been accepted by the Animal Ethics Committee at King's College London and forwarded to the Home Office Animal Project Licence for a renewal that will permit the increased number of immunisations.

Should MAbs to ω-gliadin/C-hordein peptide and rye peptide be raised, they could be tested for cross-reactivity against the selected peptides. Other MAbs could be used to determine the best combination of MAbs besides those that have been previously suggested. This could lead to the development of improved cocktail competition ELISA assays with wider specificity and greater sensitivity to quantify CD toxic proteins and peptides in commercially gluten-free foods for individuals with CD.

APPENDICES

Appendix I: Materials and Reagents

Chapter three: Investigation of CD Immunogenicity of Barley and Rye Peptides

1) Autologous serum medium (ASM)-for 50ml

   -500µl or 1/100 amphotericin B (0.25mg/ml, P11-001, PAA)

   -500µl or 1/100 HEPES buffer (H0887, Sigma)

   -50µl or 1/1000 plasmocin (25µg/ml, ant-mpt, Invivogen)

   -5ml or 1/10 autologous, heat inactivated serum (from coeliac patients)

   -44ml of RPMI 1640 with L-glutamine (PAA)

2) Organ culture medium-for 2ml

   -25µl or 1/100 amphotericin B (0.25mg/ml, P11-001, PAA)

   -25µl or 1/100 HEPES buffer (H0887, Sigma)

   -2.5µl or 1/1000 plasmocin (25µg/ml, ant-mpt, Invivogen)

   -375µl heat-inactivated foetal calf serum (PAA)

   -2ml RPMI 1640 with L-glutamine (PAA)

3) Deamidation mixture-for 500µl

   -10µl (10mg/ml) of peptic tryptic (PT) digested gluten (Roquette Ltd Corby Northants) or barley or rye (Plant Breeding International, Trumpington)

   - 135µl calcium chloride (2mM, P4417, Sigma)

   -125µl tissue transglutaminase (T5398, Sigma)

Chapter Four: Production of MAbs to immunogenic peptides of ω-glaidin/c-hordein and rye secalin

1) Gluten free diet

	g% (w/w)
Casein	20.00
Choline Bitartrate	0.25
L-Cystine	0.30
Dextrinized Starch	13.20
Corn Starch	39.75
Cellulose	5.00
Soya Oil	7.00
Sucrose	10.00
Mineral Mix	3.50
829912 AIN-93G-MX	
Vitamin Mix	1.00
829911 AIN-93-VX	
Total	100.000

2) HAT medium-for 50ml

-1 ml or 2% HAT (H0262, Sigma)

-10ml or 10% heat inactivated foetal calf serum (PAA)

-500µl or 1% Amphotericin B (PAA)

-25µl or 0.05% Plasmocin (25µg/ml, ant-mpt, Invivogen)

-38.5ml RPMI 1640 with L glutamine (PAA)

3) HT medium-for 50ml

-1ml or 2% HT (H0137, Sigma)

-10ml or 20% heat inactivated foetal calf serum (PAA)

-500µl or 1% Amphotericin B (PAA)

-25µl or 0.05% Plasmocin (25µg/ml, ant-mpt, Invivogen)

-38.5ml RPMI 1640 with L glutamine (PAA)

4) Myeloma cells medium-for 50ml

-10ml or 20% fetal calf serum (PAA)

-500µl or 1% Amphotericin (PAA)

-50µl or 0.1% Plasmocin (25µg/ml, ant-mpt, Invivogen)

-39.5ml RPMI with L-glutamine (PAA)

5) Single strength antibiotic medium (50ml)

-1000µl or 2% Penicillin/Streptomycin (5000U/ml, Gibco, Life Technologies)

-250µl or 0.5% Gentamicin (10mg/ml, G1272, Sigma)

-500µl or 1% Amphotericin B (PAA)

-48.25ml RPMI 1640 (PAA)

6) Double strength antibiotic medium (50ml)

-2000µl or 4% Penicillin/Streptomycin (5000U/ml, Gibco, Life Technologies)

-500µl or 1% Gentamicin (10mg/ml, G1272, Sigma I)

-1000µl or 2% Amphotericin B (PAA)

-46.5ml RPMI 1640 (PAA)

7) Complete medium-for 10ml

-1000µl or 10% heat inactivated foetal calf serum (PAA)

-5µl or 0.05% Plasmocin (25µg/ml, ant-mpt, Invivogen)

-9ml RPMI with L-glutamine (PAA)

8) Serum free-medium-for 10ml

-5µl or 0.05% Plasmocin (25µg/ml, ant-mpt, Invivogen)

-10ml RPMI with L-glutamine (PAA)

Chapter five: Development of cocktail competition ELISA for gluten quantification in starches

1) TCEP buffer
   -40% (v/v) Tris-HCl solution
   -0.1% TCEP reducing agent
   -60% (v/v) glycerol

2) Tris-HCl solution
   -2.422g TRIZMA Base (Sigma)
   -2.76ml of 6N HCl (Scharlau)
   -Make up with 400ml of distilled water

3) Phosphate buffer saline/ PBS tween
   -5 PBS tablet (Sigma)
   - 500µl or 0.05% of Tween 20 (VWR International Ltd)
   -1 litre of distilled water

4) Carbonate coating buffer (pH 9.6)
   -1 carbonate-bicarbonate buffer capsule (Sigma)
   -200ml of distilled water

5) Second layer detection antibody
   -1/2000 antimouse IgG or IgM alkaline phosphatase (Sigma)
   -Make up with PBS/ 0.05% Tween

Appendix II: Reagents and Equipment addresses of suppliers

Amersham Biosciences UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK

BD Plastipak, 1 Becton Drive Franklin Lakes, NJ USA 07417

Falcon, Becton Dickson Ltd, Cowley, Oxford, UK

GenScript USA Inc., 860 Centennial Ave, Piscataway, NJ 08854, USA

Harlan Teklad, Shaws Farm, Blackthorn, Bicester, Oxfordshire, OX25 1TP, UK

Health Protection Agency Culture Collections, Centre for Emergency Preparedness and Response, Porton Down, Salisbury SP4 0JG, UK

InvivoGen, Autogen Bioclear, Calne, Wiltshire, UK

LEO Laboratories Ltd, Longwick Road, Princes Risborough, Buckinhamshire, HP27 9RR, UK

PerkinElmer, Chalfont Road, Seer Green, Buckinghamshire HP9 2FX, UK

Nunc A/S, Kamstrupvej 90, Postbox 280 DK-4000 Roskilde, Denmark

PAA Laboratories, PAA-Strasse 1, 4061 Pashing, Austria

PerkinElmer, Chalfont Road, Seer Green, Buckinghamshire HP9 2FX, UK

Scientific Laboratory Supplies Ltd (SLS), Orchard House, The Square, Hessle, East Riding of Yorkshire, HU13 0AE, UK

Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset BH12 4QH, UK

Statens Serum Institut (SSI), 5Artillerivej, 2300 Coperhagen S, Denmark
VWR International-Jencons Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN, UK

Roquette Ltd Corby Northants, 22 Sallow Rd Corby, NORTHANTS NN17 5JX, United Kingdom

Plant Breeding International, Trumpington, Cambridge, UK
Transia gluten lab-test Diffchamb S.A., Lyon, France

Appendix III: Publications arise from this thesis

Papers

Widya A. Wahab, Tanja Šuligoj, H. Julia Ellis and Paul J. Ciclitira 2012, Coeliac disease immunogenecity studies of barley hordeins and rye secalins derived peptides. 26th Meeting Working Group on Prolamin Analysis and Toxicity, Leuven Belgium

Widya A. Wahab, Tanja Šuligoj, H. Julia Ellis and Paul J. Ciclitira 2013, Immunogenecity study of barley hordein and rye secalin derived peptides in gluten sensitive T cell lines of coeliac patients. 15th International Celiac Disease Symposium, Chicago, USA

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